IMAGING OF CARDIAC OUTPUT
AND REGIONAL CEREBRAL BLOOD FLOW
DURING THE MAMMALIAN DIVE RESPONSE

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Physiology
University of Saskatchewan
Saskatoon

by
Glenn P. Ollenberger
Fall 1998

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Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by
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ABSTRACT

This thesis utilized radioisotope-labeled tracers to investigate the distribution of cardiac output and regional cerebral blood flow (rCBF) during a mammalian diving response. In addition, the control mechanisms involved in the cerebrovascular response to diving were elucidated. Results indicate that in freely diving animals, the exercising front and hind limbs are not rendered totally ischemic during diving, suggesting that the demands of exercising skeletal muscle partially prevail over the peripheral vasoconstriction associated with diving. Importantly, despite a decreased cardiac output, relative blood flow to the head increased during diving, suggesting that there is a maintenance of blood flow to the brain.

The pattern of blood flow within the brain was also investigated during voluntarily initiated diving in rats. In twenty-nine of thirty-three brain regions examined, rCBF increased by an average of 1.7-fold, despite a 69.2% decrease in cardiac output. Only some regions of the basal ganglia (caudate putamen-posterior and globus pallidus) and limbic areas (hypothalamus and amygdala) did not increase rCBF significantly during diving. The overall increase in rCBF during diving was determined to be primarily due to a corresponding 20.9% decrease in cerebrovascular resistance.

Lastly, the relative contribution of humoral (carbon dioxide) and neural (trigeminal stimulation) inputs on the cerebrovasculature during a simulated dive response were explored. Pre-existing hypocapnia attenuated the increase in rCBF associated with the dive response. This result demonstrated that the decrease in cerebrovascular resistance during diving in small mammals is driven primarily by the
progressive hypercapnia associated with asphyxia. Furthermore, the results from this study suggest that trigeminal input does not play any role in differentially modulating the cerebrovascular response to diving.

It is concluded that an oxygen conserving response, involving a redistribution of blood flow toward the brain, occurs during voluntarily initiated diving in rats. Although exercising skeletal muscle was shown to modify the peripheral distribution of blood flow during diving, the brain's share of cardiac output increases primarily due to a decrease in cerebrovascular resistance. The resultant increase in CBF during diving was demonstrated to be primarily driven by the progressive hypercapnia associated with asphyxia.
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DEDICATION

For Leanne and Oliver.
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<th>Description</th>
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<tr>
<td>BH</td>
<td>breath-hold</td>
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<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
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<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CVR</td>
<td>cerebral vascular resistance</td>
</tr>
<tr>
<td>DMRF</td>
<td>dorsal medullary reticular formation</td>
</tr>
<tr>
<td>H⁺</td>
<td>hydrogen ion</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>bicarbonate</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>ICF</td>
<td>intracellular fluid</td>
</tr>
<tr>
<td>IMP</td>
<td>$^{14}$C-N-isopropyl-p-iodoamphetamine</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>MABP</td>
<td>mean arterial blood pressure</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ion</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>NO-synthase</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus of the solitary tract</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>arterial carbon dioxide partial pressure</td>
</tr>
<tr>
<td>PaO₂</td>
<td>arterial oxygen partial pressure</td>
</tr>
<tr>
<td>rCBF</td>
<td>regional cerebral blood flow</td>
</tr>
<tr>
<td>ROI</td>
<td>regions of interest</td>
</tr>
<tr>
<td>SV</td>
<td>stroke volume</td>
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<tr>
<td>$^{99m}$Tc-ECD</td>
<td>technetium-99m ethyl cysteinate dimer</td>
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<tr>
<td>TPR</td>
<td>total peripheral resistance</td>
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1.0. GENERAL INTRODUCTION.

1.1. INTRODUCTION.

In mammals, brain tissue is undeniably the most sensitive of all tissues to oxygen deprivation. If blood flow to the brain is compromised, even for a short time, loss of consciousness or irreversible neurological injury can occur. In the rat, cortical activity ceases after only two minutes of complete oxygen deprivation (Sick et al., 1982). This emphasizes the importance of the control mechanisms in the cerebral circulation that maintain blood flow and oxygen delivery to this vital organ.

Although, mammals are highly adaptive to their environment, the innate vulnerability of such air-breathing animals is immediately evident during episodes of apnea. One naturally occurring condition of prolonged apnea is during breath-hold (BH) diving. It has been reported in many studies that some mammals can withstand periods of underwater submergence for longer periods of time than would be expected based on oxygen storage (for a review, see Butler and Jones, 1982). This remarkable ability to extend underwater duration appears to be due primarily to a cardiovascular adjustment termed the "diving response." It has been proposed, for some time, that the dive response redistributes oxygenated blood flow away from tissues with a capacity
for anaerobic metabolism, such as skeletal muscle, toward the oxygen sensitive heart and brain (Irving, 1934; Johansen, 1964). However, recent studies in freely diving animals suggest that exercising muscle may not be deprived of oxygen during diving as previously thought (Butler et al., 1988; Bevan and Butler, 1992). Moreover, within the brain itself, blood flow is thought to be at least maintained. However, few studies have attempted to study blood flow within the brain during diving. In this thesis the distribution of regional cerebral blood (rCBF) and cardiac output (CO) were investigated in a freely diving small mammal – the rat. In addition, the control mechanism involved in the cerebrovascular response to diving were elucidated.

Before passing to the specific objectives of this thesis, a review of the principal levels of control that are involved in the regulation of cerebral blood flow (CBF) will be presented. Following this will be a brief discussion of the cardiovascular and cerebrovascular adjustments during the mammalian diving response. Lastly, a review of the rationale for investigating the distribution of cardiac output and rCBF during diving in the rat will be presented.

1.2. THE REGULATION OF CEREBRAL BLOOD FLOW.

The cerebral tissue has a limited ability for anaerobic metabolism during conditions of decreased oxygen delivery. Therefore, the brain is highly intolerant to a reduction in its blood supply. There are a number of mechanisms in place that ensure
blood flow, and therefore oxygen delivery, is maintained to this highly oxygen sensitive tissue.

In the context of this thesis, the regulation of CBF can be viewed as occurring at three distinct levels of control: neural, metabolic and systemic. Neural control refers to the influence of nerves, such as autonomic innervation, on the cerebrovasculature. Metabolic control of CBF occurs as a result of the local production of vasoactive mediators from metabolically active brain tissue. Lastly, systemic control refers to the influence of both cardiovascular and respiratory variables upon the control of CBF. These three levels of CBF control are discussed below.

1.2.1. **Neural control of cerebral blood flow.**

Three distinct types of perivascular nerves have been described in the cerebrovascular system: sympathetic, parasympathetic and sensory (trigeminal) nerve fibers (for a review see, Edvinsson et al., 1993; Goadsby and Sercombe, 1996). In addition there is a network of nerves within the brain itself, known as intrinsic innervation, that can modulate CBF.

1.2.1.1. **Sympathetic nerve fibers.**

Sympathetic fibers to the brain originate in the superior cervical ganglia with fewer nerve fibers originating from the stellate ganglion (Iwayama et al., 1970). These
ganglia contribute fibers to the cerebral vessels by travelling along the internal carotid and vertebral arteries respectively. Sympathetic fibers have been demonstrated to contain norepinephrine (Nielsen and Owman, 1967), neuropeptide Y (NPY) (Edvinsson et al., 1983), and serotonin (5-HT) (Griffith et al., 1982) in their nerve terminals. Although the sympathetic fibers are distributed to vessels throughout the cerebral circulation, the greatest density of innervation is to the circle of Willis and the major anterior vessels (Arbab et al., 1986). Furthermore, it is clear that there is a regional heterogeneity in the extent of innervation to small brain divisions. Edvinsson and Owman (1977) examined brain divisions in the baboon and found that the medial geniculate and inferior colliculus had the greatest density of sympathetic innervation, whereas the occipital cortex and cerebellum had the least innervation. The degree of innervation to the cerebrovasculature most likely produces the variability in the CBF response during sympathetic stimulation (Tuor, 1989).

Generally, the sympathetic system has little tonic or resting vasoconstrictor influence upon the cerebral circulation (Heistad et al., 1978). Instead, the sympathetic system seems to be important in modifying the autoregulatory response of the cerebral circulation to arterial pressure (see Section 1.2.3.1. "The cardiovascular system and the regulation of cerebral blood flow."). In addition, recent evidence suggests that the sympathetic system may be involved in limiting the increase in CBF that occurs during both hypercapnia and hypoxia. Busja and Heistad (1984) demonstrated in cats and rabbits that sympathetic stimulation attenuated the increase in CBF during hypercapnia. In rats, sympathectomy resulted in higher rates of CBF during hypoxia compared to controls (Kissen and Weiss, 1989). The above studies suggest that the
sympathetic system appears to play an important role in limiting an increase in CBF in response to a profound decrease in cerebrovascular resistance (CVR).

1.2.1.2. Parasympathetic nerve fibers.

The origins of the parasympathetic vascular innervation to the brain have been elucidated through retrograde axonal tracing studies. The primary source of preganglionic nerves originates in the superior salivatory nuclei in the pons and traverses through the facial nerve (VIIth cranial nerve) to the sphenopalatine and otic ganglia (Goadsby and Sercombe, 1996). From these ganglia, postganglionic parasympathetic nerves innervate the cerebral blood vessels. Biochemical studies have revealed that parasympathetic nerves contain acetylcholine (ACh) (Florence et al., 1979), vasoactive intestinal peptide (VIP) (Larsson et al., 1976) and peptide histidine isoleucine (PHI) (Edvinsson and McCulloch, 1985).

Electrical stimulation of the facial nerve increases CBF as measured by both laser Doppler flowmetry and iodoantipyrine in cats (Goasby, 1989; Goadsby, 1991). The response to facial nerve stimulation has been shown to be a purely neurogenic response since the increase in CBF is not associated with an increase in metabolism (Goadsby, 1989). In addition, electrical stimulation of the sphenopalatine ganglion produces a 50% increase in CBF in the parietal cortex in the rat (Seylaz et al., 1988). The physiological significance of these induced CBF changes remains uncertain, since cholinergic blockade does not affect resting CBF (Suzuki and Hardebo, 1993). The parasympathetic pathway does not play any role in modulating the CBF response to
hypercapnia. In anesthetized cats, application of atropine, a parasympathetic blocker, does not attenuate the increase in CBF associated with hypercapnia (Busija and Heistad, 1982). Furthermore, sectioning of the facial nerve does not alter the cerebrovascular reactivity to hypercapnia (Goadsby, 1991). These fibers may, however, contribute to cerebral autoregulation during decreasing perfusion pressure (see Section 1.2.3.1. "The cardiovascular system and the regulation of cerebral blood flow.").

1.2.1.3. Sensory nerve fibers.

Sensory nerve fibers include those nerves that are responsive to external stimuli and have neurons in sensory ganglia. Generally, any reference to sensory nerve fiber innervation in the cerebral circulation refers to the trigeminal system (cranial nerve V). The neural connections of the trigeminal system with cerebral blood vessels are so numerous, that the concept of a trigeminocerebrovascular system has arisen (Moskowitz, 1984). The trigeminal ganglion contains bipolar cells that make connections peripherally, primarily through the ophthalmic branch (V₁) of the trigeminal nerve to the cerebral vessels, and centrally, to the spinal tract of the trigeminal nerve in the lower medulla (Suzuki et al., 1989).

There appears to be two distinct peptides that are released in the cerebral circulation upon sensory stimulation: substance P (SP) and calcitonin gene-related peptide (CGRP). These two peptides have been found to exist in dense networks of
nerve fibers surrounding cerebral arteries and veins and are potent vasodilators (Edvinsson et al., 1981; McCulloch et al., 1986)

Electrical stimulation of the trigeminal nerve or ganglion has been shown to produce regional variations in CBF (Goadsby and Duckworth, 1987) and decrease carotid arterial resistance (Lambert et al., 1984). The alterations in CBF have been demonstrated to be limited to the frontal and parietal cortices without affecting deeper cerebral structures. The physiological role of the trigeminovascular system, however, remains obscure. It has been speculated that it responds to hypoxic insults and seizures (Goadsby and Sercombe, 1996). Sectioning of trigeminal innervation to the brain does not affect resting CBF indicating that sensory nerve fibers do not contribute to the regulation of resting blood flow (Suzuki et al., 1990). Pathophysiologically, there is evidence to suggest that the trigemino-cerebrovascular system is involved in the manifestation of migraine headaches (Moskowitz et al., 1988). Release of the vasoactive neuropeptide, CGRP, during a migraine, is antagonized by antimigraine agents that return CBF to control levels (Goadsby and Edvinsson, 1993).

1.2.1.4. Intrinsic neural innervation.

The concept that activation of discrete brain regions can influence rCBF is not new (Stavrasky, 1936). Stavrasky demonstrated that electrical stimulation of anatomically undefined areas within the rostral medulla could modify CBF independent of arterial pressure and sympathetic nerve activity. Since then, a number of studies have been undertaken that stimulate specific regions of the brain,
electrically or chemically, and measure the response of rCBF. The following discussion will be limited to brainstem regions, since these regions are thought to be necessary for the integration of the diving response (Blix and Folkow, 1984). The reader is directed to expert reviews for comprehensive treatises on the other known brain regions involved in the intrinsic regulation of CBF (Iadecola, 1992; Reis and Iadecola, 1989; Mraovitch, 1996).

C1 Area. Neurons in the C1 area are principally adrenergic and provide tonic input to the peripheral sympathetic nervous system (Reis and Iadecola, 1989). The C1 area of the rostral ventrolateral medulla is now recognized as a major brainstem nucleus governing arterial blood pressure (Reis et al., 1988). In addition, this region appears to strongly influence the cerebral circulation. In rats, electrical stimulation of the C1 area elevated arterial pressure and increased rCBF two to threefold bilaterally and symmetrically throughout the whole brain (Underwood et al., 1992). Furthermore, it has been shown that the C1 mediated increase in rCBF is not associated with a increase in global brain metabolism (Underwood et al., 1992).

Nucleus of the solitary tract (NTS). The NTS is the principal site of integration of cardiovascular and respiratory afferents (Andresen and Kunze, 1994). Lesioning of the NTS elicits a potent elevation in arterial pressure, known as NTS-hypertension (Doba and Reis, 1973). Electrical stimulation of the NTS has also been demonstrated to increase CBF globally in rats (Nakai, 1985). Interestingly, lesioning the NTS
impairs cerebral autoregulation globally, suggesting that the NTS has control over CBF by an unknown mechanism (Ishitsuka et al., 1985).

**Dorsal medullary reticular formation (DMRF).** The DMRF region resides partially within the parvocellular reticular nucleus in the brainstem. Electrical stimulation of the DMRF elicits an increase in CBF that involves the entire brain, occurring maximally (2.4-fold) in the cerebral cortex (Iadecola et al., 1983). The DMRF driven increase in CBF appears to be tightly coupled to increased metabolism (Iadecola et al., 1983), suggesting that the vasodilation is likely a result of the increased metabolic activity. Curiously, the cerebrovasodilation elicited by the DMRF is dependent on the adrenal glands, since adrenalectomy significantly attenuates the response (Iadecola et al., 1987).

1.2.2. *Metabolic control of cerebral blood flow.*

Metabolic control of CBF refers to the response of cerebral blood vessels to vasoactive mediators produced in the immediate surrounding of blood vessels. The primary physiological role of the cerebral circulation is to provide a continuous supply of oxygen and glucose for the generation of adenosine triphosphate (ATP). Increased neural activity is characterized by an increase in the frequency of action potentials, that depletes ATP. Thus, an increase in neural activity requires an increased delivery of energy substrate and oxygen through the blood. Therefore, it is logical for the products
of cerebral tissue metabolism to increase local CBF, thereby delivering the required 
energy substrate. This concept is not new and began with Roy and Sherrington’s 
(1890) classical hypothesis:

“The chemical products of cerebral metabolism contained in the 
lymph which bathes the walls of the arterioles of the brain can cause 
variations of the caliber of the cerebral vessels. In this reaction the brain 
possesses the intrinsic mechanism by which its vascular supply can be 
varied locally in correspondence with local variations of functional 
activity.”

This hypothesis could not be proven until the development of modern 
techniques for measurement of rCBF and metabolism. The development of local 
cerebral glucose utilization (lCGU) and local regional blood flow measurements 
enabled investigators to determine, for the first time, regional changes in the brain 
during neural activity. These techniques utilized autoradiography for tracer estimation 
in tissue and clearly demonstrated that the pattern of lCGU corresponded to the 
pattern of rCBF, thus indicating a coupling between them (Figure 1.1). Moreover, it 
appeared that glucose delivery closely paralleled blood flow. This was not surprising, 
since blood is the medium that delivers glucose to the target tissue.

Recent studies have also demonstrated an apparent coupling of glucose 
delivery and blood flow to neurally active brain regions. This evidence has been 
determined primarily through positron-emission tomography (PET). PET studies 
utilize radioisotopes and computerized tomography to reveal the dynamic distribution 
of neural activity and blood flow within the brain. Greenberg et al. (1981) used $^{18}$F-
deoxyglucose to follow regional changes in cerebral glucose metabolism during
Figure 1.1. Correlation between local cerebral glucose utilization and local cerebral blood flow in conscious rats. The relationship between glucose metabolism and cerebral blood flow was linear with a strong correlation ($r^2 = 0.96$). [From Kuschinsky et al. (1981)].
auditory stimulation, and demonstrated that glucose delivery quickly matched brain
activity. Regional CBF has also been shown, through PET scans, to closely match
neural activity. During talking, regions of the brain known to be involved in speech
were shown to receive an increased rCBF (Ingvar, 1976). Other experimental
paradigms, utilizing autoradiography, have also demonstrated that neural activity is
matched to both rCBF and ICGU. During auditory stimulation in the conscious rat,
rCBF and ICGU increased to the neuroanatomic components of the primary auditory
system, suggesting a coupling of rCBF and ICGU to neural activity (Edvinsson et al.,
1993). Overall, these studies demonstrate that a strong coupling exits between
neurally active brain regions and local cerebral metabolism and blood flow in the
brain.

Since these studies, numerous metabolites have been investigated for their role
in coupling CBF to local metabolic activity. Before the potential mediators that couple
rCBF to metabolic activity are discussed a brief review of the mechanisms underlying
neural activity and metabolism will be presented.

1.2.2.1. Neural activity and metabolism.

An increase in neural activity can be viewed as an increase in the frequency of
action potentials elicited by a neuron. An action potential requires an initial resting
membrane potential that is determined primarily by the concentration gradient of ions,
particularly sodium (Na⁺) and potassium (K⁺), across the cell membrane. When an
action potential is elicited, Na⁺ permeability changes and Na⁺ rapidly diffuses from the
extracellular fluid (ECF), where its concentration is high, to the intracellular fluid (ICF), where its concentration is low. The movement of Na\(^+\) from the ECF to the ICF results in membrane depolarization. Following depolarization, the cellular membrane is repolarized by the movement of K\(^+\) from a high concentration within the cell, to a low concentration in the ECF. However, the ionic potential of the resting membrane must be restored in order for another action potential to be elicited. This requires an ATP dependent Na\(^+\)/K\(^+\) pump that pumps Na\(^+\) out of the cell in exchange for K\(^+\). The pump itself hydrolyzes ATP to produce hydrogen ion (H\(^+\)) and adenosine monophosphate (AMP). The ATP pump is the single most important determinant of ATP utilization, the primary storage of energy in the cell. This was demonstrated by Astrup et al. (1981) in which inhibition of Na\(^+\)/K\(^+\) ATPase, the enzyme needed for ATP hydrolysis that drives the pump, produced a 80% reduction in glucose utilization within the brain. This suggests that approximately 80% of all energy generated by brain tissue is destined to be used for maintenance of ionic gradients. The utilization of ATP to drive active transport, requires that ATP must be regenerated, ultimately by burning glucose. A major production pathway of ATP synthesis is through oxidative phosphorylation, a metabolic pathway that requires oxygen. Within the brain, 90% of total ATP production from glucose is from this pathway (Edvinsson et al., 1993). Therefore, oxygen is essential for ATP production. It is not surprising, therefore, that neurons are responsible for 75% of all oxygen consumed by the central nervous system (Siesjö, 1978).

1.2.2.2. Carbon dioxide and hydrogen.
Both carbon dioxide (CO₂) and H⁺ increase in concentration during neural activity and metabolism. H⁺ increases due to activation of the ATP driven membrane pump and CO₂ increases due to increased cellular respiration as a result of ATP utilization. Thus, H⁺ from ATP hydrolysis is linked directly to neural activity, while CO₂ production is linked indirectly, since it is only produced as a result of increased metabolism following neural activity. Both CO₂ and H⁺ have been shown to have pronounced vasodilatory effects on cerebral blood vessels (for a review, see Heistad and Kontos, 1983). However, CO₂ is most likely not the direct mediator of the vasomotor change, instead, it is the CO₂ derived change in H⁺ concentration in the extracellular fluid (Kontos et al., 1977). This relationship is evident from the hydration equation for carbon dioxide:

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- \tag{1.1}
\]

Therefore, as CO₂ is produced during increased metabolic activity, the concentration of H⁺ also rises.

Although H⁺ has been shown experimentally to vasodilate cerebral vessels, evidence suggests that H⁺ is unlikely to couple rCBF to metabolism. Leninger-Follert (1984) demonstrated that rCBF increases before any increase in H⁺ concentration during neural activity. Furthermore, H⁺ concentration does not increase despite a three-fold increase in CBF during amphetamine induced neural activation (Berntman et al, 1978). Therefore, a change in H⁺ concentration can induce cerebrovascular
vasomotor changes, but a role for $H^+$ in coupling $\text{rCBF}$ to local metabolism seems unlikely. However, even if $H^+$ does not link $\text{rCBF}$ to local metabolism, the response of cerebral blood vessels to changes in $H^+$ concentration cannot be overlooked. This concept will be discussed in Section 1.2.3.2. “The respiratory system and the regulation of cerebral blood flow.”

1.2.2.3. Oxygen.

During increased neural activity, there is an increase in aerobic metabolism that depletes cellular oxygen. The decrease in cellular oxygen was thought to lead to a decrease in local oxygen levels. A decrease in local oxygen has been shown to cause vasodilation of cerebral blood vessels in numerous studies (Lombard et al., 1986; Vinall and Simeone, 1986). However, in a recent study, brain deoxyhemoglobin concentration decreased within seconds of neural activity, indicating that increased metabolic activity leads to increased local oxygen levels not decreased levels (Ogawa et al., 1992). Furthermore, local cortical oxygen tension was elevated during a seizure-induced increase in brain activity (Leninger-Follert, 1984). The results from these studies suggest that during increased neural activity, local tissue oxygenation is maintained or increased, most likely by local increases in $\text{rCBF}$. Therefore, it is unlikely that a decrease in local oxygen tension levels couples $\text{rCBF}$ to neural metabolism.

1.2.2.4. Potassium ions.
During neuronal activity, as the cell repolarizes, $K^+$ is released into the ECF where its concentration rises. Cerebral arteries increase in diameter by as much as 50% to a change in $K^+$ concentration of this magnitude (Kuschinsky et al., 1972). Therefore, it is possible that when $K^+$ is released into the ECF, as during repolarization, it diffuses toward cerebral blood vessels and decreases vascular resistance.

Astrocytes are also a potential source of $K^+$ during neuronal activity. Interestingly, cerebral arteries are completely surrounded by the endfeet of astrocytes. Paulson and Newman (1987) suggested that $K^+$ may be taken up by astrocytes during repolarization and then transported out the astrocytic endfeet near cerebral blood vessels. Thus, it is possible that during increased neuronal activity, $K^+$ is released into the ECF and “siphoned” toward cerebral arteries by astrocytes. However, neuronal activity induced by amphetamine administration is accompanied by a three-fold increase in blood flow without any associated changes in the extracellular levels of $K^+$ (Berntman et al., 1978), suggesting that $K^+$ does not play an important role in the coupling of rCBF to metabolism.

1.2.2.5. Adenosine.

Adenosine is the metabolic by-product of ATP consumption during neuronal activity. Adenosine is a potent vasodilator of cerebral blood vessels and may play a role in coupling rCBF to metabolic activity (Phillis, 1989; Winn et al., 1981).
Dirnaghl et al. (1994) found that adenosine receptor blockade reduced the rCBF response to somatosensory stimulation by approximately 40%. Other investigators have demonstrated that adenosine antagonists do not attenuate cerebral vasodilation associated with hypoxia or electrical stimulation (Dora et al., 1980; Northington et al., 1992). Brain adenosine concentration has been demonstrated to increase two-fold during the first 5 seconds following ischemia; a concentration change that would only have a small effect on vessel diameter (Wahl and Kuschinsky, 1977).

1.2.2.6. Nitric Oxide.

Recently, the role of nitric oxide (NO) in the regulation of the cerebral circulation has been the focus of much attention (for reviews, see Faraci and Brian, 1994; Iadecola et al., 1994; Brian et al., 1996). NO is produced from L-arginine by the action of the enzyme NO-synthase (NOS) (Palmer et al., 1988). Once NO is generated, it freely diffuses into the cytosol of smooth muscle cells and elicits a vasodilatory response. The source of NO was previously believed to be limited to the endothelium. However, in the brain, NOS has been found to be located not only in endothelium, but also in neurons and perivascular nerves (Bredt et al., 1990). Results from studies utilizing NOS blockers in an attempt to attenuate metabolically driven cerebrovasodilation have been variable (for review see Iadecola et al., 1994).

Some studies report NO to have a significant role in the control of CBF during neuronal activity (Dirnaghl et al. 1993; Irikura et al., 1994). These investigators attenuated the rCBF response to somatosensory stimulation in the cerebral cortex of
the rat by inhibiting NOS. In addition, Irikura et al. (1994) found a correlation between the degree of NOS inhibition and the attenuation of the rCBF response. Other studies have not been able to demonstrate that NO couples CBF to neural activity (Adachi et al., 1994; Wang et al., 1993). These investigators inhibited NOS before somatosensory stimulation in the rat and found that the associated increase in rCBF was not mediated by NO. The studies to date are conflicting, and the precise role for NO in coupling CBF to neural activity remains to be determined.

1.2.3. The influence of the cardiovascular and respiratory systems on cerebral blood flow.

Although the majority of control in the cerebral circulation, as discussed above, occurs within the brain itself, these control mechanisms must operate in association with the properties of the entire circulation. Alterations in the hemodynamics of the cardiovascular system potentially result in corresponding changes within the cerebral circulation. The respiratory system also has a significant effect upon CBF. In particular, arterial blood gases, such as carbon dioxide and oxygen, can elicit significant changes in CBF. The influence of both cardiovascular and respiratory variables on the cerebral circulation will be discussed below.

1.2.3.1. The cardiovascular system and the regulation of cerebral blood flow.
Since the blood supply to the cerebral circulation is derived from the systemic circulation, it is logical that systemic circulatory variables influence CBF. From Ohm's law, mean arterial blood pressure (MABP; mmHg) is equal to cardiac output (CO; ml/min) multiplied by the total peripheral resistance (TPR; mmHg/ml/min):

\[ \text{MABP} = \text{CO} \times \text{TPR} \tag{1.2} \]

where TPR is the sum of all the individual resistances within the entire systemic vascular bed. The cerebral circulation has a resistance, CVR, that is in parallel with the resistance of other tissues \((R_1, R_2, \ldots, R_n)\):

\[ \text{CO} = \frac{\text{MABP}}{\text{CVR} + R_1 + R_2 + \ldots + R_n} \tag{1.3} \]

Furthermore, cardiac output represents the sum of all flows within the circulation \((Q_1, Q_2, \ldots, Q_n)\), including CBF:

\[ \text{CO} = \text{CBF} + Q_1 + Q_2 + \ldots + Q_n \tag{1.4} \]

Substituting equations 1.3 and 1.4 into Equation 1.2 results in:

\[ \text{CBF} + Q_1 + Q_2 + \ldots + Q_n = \frac{\text{MABP}}{\text{CVR} + R_1 + R_2 + \ldots + R_n} \tag{1.5} \]
Equation 1.5 shows that the cerebral circulation is in parallel with the entire circulatory system. Therefore changes in flow or resistance in tissues parallel to the cerebral circulation potentially modulate CBF. If resistance and blood flow to all parallel tissues remains constant then:

\[
\text{CBF} = \frac{\text{MABP}}{\text{CVR}}
\]  

Therefore, blood flow within the cerebral circulation is directly related to arterial pressure and indirectly related to cerebral vascular resistance. However, CBF has been shown to be relatively constant over a wide range of arterial pressure primarily due to changes in CVR – a phenomenon known as autoregulation. The concept of autoregulation will be discussed below.

**Arterial pressure and cerebral autoregulation.** Changes in perfusion pressure do not normally result in alterations in CBF due to the ability of the cerebral circulation to autoregulate (Kuschinsky, 1988). Generally, the autoregulatory mechanism keeps CBF constant by vasodilating in response to decreased arterial pressure, and vasoconstricting in response to increased arterial pressure. Autoregulation of CBF is effective over a wide range of arterial blood pressures, but has both a lower and an upper pressure limit (Figure 1.2). In the rat, the lower and upper limits of autoregulation are approximately 50 and 150 mmHg, respectively (Hernandez et al., 1978). Below the lower limit, a decrease in arterial pressure is matched by a decrease in CBF. Above the upper limit, CBF increases linearly with arterial pressure.
Figure 1.2. Cerebral blood flow (CBF) versus mean arterial blood pressure (MAP). Cerebral blood flow remained constant over a wide range of arterial pressures. However, there was both an upper and lower limit of autoregulation. Below the lower limit, a decrease in MAP was matched by a decrease in CBF. Above the upper limit, CBF increased linearly with MAP [From Edvinsson et al. (1993)].
CBF autoregulation has been shown to be modified by various external stimuli, including neural input and arterial carbon dioxide (Paulson et al., 1990). An increase in the upper limit of autoregulation occurs during sympathetic stimulation (Barry et al., 1982), suggesting that sympathetic activation buffers CBF to acute increases in arterial blood pressure. Shimizu et al. (1991) demonstrated that cerebral dilation is abolished after parasympathetic nerve section in the rat during hypotension. This suggests that parasympathetic innervation modulates CBF during decreased systemic arterial pressure. During hypercapnia, when CBF is increased, cerebral blood vessels lose the ability for autoregulation at lower arterial pressures (Paulson et al., 1990). Therefore during hypercapnia the upper limit of autoregulation may be lowered, resulting in linear increases in CBF at lower than normal arterial pressures. Hypocapnia has the opposite effect; the upper limit of autoregulation is elevated. Therefore during hypocapnia, when CBF is decreased, the cerebral vessels may have an increased ability to compensate for higher arterial pressures (Paulson et al., 1990).

1.2.3.2. The respiratory system and the regulation cerebral blood flow.

The primary role of the respiratory system is to remove carbon dioxide from venous blood and replenish arterial oxygen. Generally, the arterial partial pressures of oxygen (PaO₂) and carbon dioxide (PaCO₂) are held relatively constant by mechanisms that control ventilatory drive, such as chemoreceptors. However, PaO₂ and PaCO₂ rapidly change in response to asphyxia due to the termination of respiratory gas exchange with the environment. As a result, during asphyxia, PaO₂ progressively falls,
while at the same time $P_{aCO_2}$ rises. As will be evident from the following discussion, alterations in arterial blood gases potentially influence CBF.

**Arterial carbon dioxide.** It is generally accepted that cerebrovascular resistance is highly sensitive to changes in $P_{aCO_2}$. Numerous studies have clearly demonstrated that systemic hypercapnia elicits a marked vasodilation in the cerebral circulation (for a review, see Edvinsson *et al.*, 1993). In almost all studies CBF increases steeply in response to increased $P_{aCO_2}$. The reactivity of CBF in response to $P_{aCO_2}$ has been described by a sigmoid curve with the linear portion ranging between approximately 25 to 70 mmHg (Figure 1.3) (Harper and Glass, 1965; Olesen *et al.*, 1971; Reivich, 1964). The reactivity of the cerebrovasculature to CO$_2$ is most likely mediated by the CO$_2$-driven change in ECF pH (Kontos *et al.*, 1977). Since CO$_2$ diffuses freely across the blood-brain barrier (BBB) the arterial levels of CO$_2$ significantly effect ECF pH in the brain. However, alterations in arterial pH do not effect CBF, since the BBB is relatively impermeable to H$^+$ (Harper and Bell, 1963).

Hypocapnia decreases CBF, however, the reactivity to a fall in $P_{aCO_2}$ to is not as great as to hypercapnia. Hyperventilation, which decreases $P_{aCO_2}$, reduces CBF by a maximum of 30% to 50% (Reivich, 1964). The probable mechanism is a concomitant bicarbonate (HCO$_3^-$) increase in the brain ECF (Cameron and Caronna, 1976). However, since the BBB is relatively impermeable to HCO$_3^-$, arterial HCO$_3^-$ changes do not effect CBF (Lambertsen *et al.*, 1961).
Figure 1.3. The percent change in cortical blood flow versus the concentration of arterial CO₂ in dogs. Cortical blood flow increases steeply in response to an increase in arterial CO₂. A decrease in arterial CO₂ below approximately 20 mmHg does not result in a decrease in CBF, whereas an increase above approximately 80 mmHg does not result in corresponding increase in CBF [From Harper and Glass, (1965)].
Figure 1.4. The relationship between cortical blood flow (expressed as percentage of control) and the arterial pressure of oxygen (PaO₂) in dogs. Cortical blood flow was virtually unchanged over a PaO₂ range of 55 to 140 mmHg, at constant Paco₂. However, cortical blood flow increased sharply below approximately 55 mmHg, suggesting that a threshold must be reached before CBF reacts to the low PaO₂ [From McDowall and Harper, (1968)].
Arterial oxygen. The reactivity of the cerebrovasculature in response to hypoxia is significantly less than to hypercapnia (McDowall and Harper, 1968; James et al., 1969). In fact, CBF has been shown to be virtually unchanged over a PaO₂ range of 55 to 140 mmHg, at constant PaCO₂ (Figure 1.4) (McDowall and Harper, 1968). However, CBF increases sharply below approximately 55mmHg, suggesting that a threshold must be reached before CBF reacts to the low PaO₂. Conversely, an increase in PaO₂ has also been shown to produce modest cerebral vasoconstriction (Kontos et al., 1978). Therefore, the above studies suggest that the overall reactivity of the cerebrovasculature to PaO₂ is significantly less than that of PaCO₂.

1.3. THE MAMMALIAN DIVING RESPONSE.

The mammalian diving response has three main components: apnea, bradycardia and peripheral vasoconstriction. The diving response has been extensively reviewed by a number of investigators (Blix and Folkow, 1984; Butler and Jones, 1882; Elsner and Gooden, 1983; Hochachka, 1980; Kooymen, 1989). This thesis will not review all of the components of the mammalian diving response, but rather provide an overview of the cardiovascular and cerebrovascular adjustments to diving. Specific reference will also be made to studies in ducks where appropriate. The reader is directed to these reviews for comprehensive reviews on diving.
1.3.1. *Cardiovascular adjustments during diving.*

A profound decrease in heart rate is the most prominent feature of the cardiovascular response to diving. In fact, the change in heart rate is so obvious that it was discovered by feeling the heart beat through the breast of a submerged duck (Bert, 1870). Bradycardia occurs upon submersion in many aquatic and terrestrial mammals, including man (Lin, 1982) with little change in stroke volume (Lin and Baker, 1975). Since cardiac output is equal to stroke volume multiplied by heart rate, there is a resultant decrease in cardiac output that is directly proportional to the decrease in heart rate. From Equation 1.2 it is clear that unless TPR increases during diving, MABP will fall in response to the decreased cardiac output. However, arterial blood pressure remains relatively unchanged during diving (Jones et al., 1982; McCulloch et al. 1997), suggesting that TPR increases to match the decrease in cardiac output. The increased vascular resistance during diving results from an increase in sympathetic outflow to the peripheral vascular beds (Bron et al., 1966). From Equation 1.5, if resistance is increased to tissues in parallel with the systemic circulation, the tissues will receive a reduced fraction of cardiac output. The increase in TPR would redirect blood flow toward tissues with lower resistance during diving, such as the brain. Therefore, blood flow to the brain can be maintained during diving, provided perfusion pressure remains relatively constant. In addition, if CVR decreases during diving, then a corresponding increase in CBF can occur (see Equation 1.6).
overall result of the circulatory adjustments during diving is to redistribute a
decreased cardiac output toward the brain (Lin and Baker, 1975; Zapol et al., 1979).
The redistribution of cardiac output during diving will be the focus of the following
discussion.

Johansen (1964) was likely the first to examine the regional distribution of
cardiac output during diving. The results from this pioneering study suggested that
blood flow to most peripheral organs was effectively shut down during forced head
immersion in the duck. Notable exceptions to the ischemia during diving were the
brain, heart and adrenal glands. More recent investigations in ducks have revealed
similar results in both absolute (Jones et al., 1979) and relative terms (Heieis and
Jones, 1988).

Investigations in mammals have demonstrated that there is a profound
redistribution of cardiac output during forced submersion. In seals, the liver, kidney,
stomach and skeletal tissues are rendered essentially ischemic during diving (Blix et
al., 1976; Blix et al., 1983; Zapol et al., 1979). In muskrat (Jones et al., 1982), beaver
(McKean, 1982) and rat (Lin and Baker, 1975) the redistribution of cardiac output
during forced submersion appears to parallel the changes seen in more specialized
divers. In the muskrat, relative blood flow, a measure of tissue blood flow in relation
to a decreased cardiac output, only increased to the heart and brain, while all others
regions received a decreased blood flow during diving (Figure 1.5). However, it must
be emphasized that an increase in relative blood flow does not necessarily represent an
absolute increase if it is accompanied by a decrease in cardiac output. In the rat and
beaver, blood flow, in absolute terms, decreased to most tissues during diving except
Figure 1.5. Distribution of cardiac output during forced diving in muskrats. The percentage of cardiac output going to a range of tissues changed markedly during the dive [From Jones et al., (1982)].
Figure 1.6. Direct measurement of blood flow through the ischiadic arteries (to the hindlimbs) during voluntary diving in ducks. During diving, blood flow to the hindlimbs increased markedly above resting values (R) and was maintained near predive values towards the end of the dive [From Bevan and Butler, (1992)].
for the heart and brain (Lin and Baker, 1975; McKean, 1982). Overall, the above studies demonstrate that blood flow to peripheral tissues is effectively shut down and shunted toward the more oxygen dependent heart and brain.

However, most studies have examined the distribution of cardiac output in restrained animals in which there is no locomotory activity. Freely diving animals have exercising muscle that may possibly demand additional blood flow. In fact it has been proposed (Butler, 1982; Millard et al., 1973) that in birds, the cardiovascular adjustments made during voluntary diving may be a composite of the exercise response, where blood flow is redistributed in favor of skeletal muscle (Butler et al., 1988), and the forced dive response, where blood flow to hypoxia-insensitive tissues is effectively shut down (Jones et al., 1979). In support of this view, the distribution of blood flow has been examined in freely diving ducks by both direct (Bevan and Butler, 1992) and indirect measurements (Stephenson and Jones, 1992). Blood flow to the active leg muscles increased nearly fivefold compared to control levels in voluntary diving ducks (Bevan and Butler, 1992) (Figure 1.6). Stephenson and Jones (1992) found that the distribution of blood flow in freely diving ducks was highly variable and depended upon the dive type. During escape diving in ducks, in which the hindlimbs were active, relative blood flow to the hindlimbs increased significantly compared to the values in resting ducks. However, during forced diving, in which there was no locomotory activity, relative blood flow to the hindlimbs decreased moderately compared to the values in resting ducks (Stephenson and Jones, 1992). The above studies on ducks demonstrate that the circulatory adjustments during diving change in response to the demands of locomotory activity.
1.3.2. *Cerebrovascular adjustments during diving.*

Although previous studies suggest that the brain is perfused continuously during diving, few studies have examined the cerebral circulation during diving. A modification of the Fick equation shows that an increase in CBF during diving can maintain oxygen delivery in the face of progressive hypoxemia:

\[
\text{Cerebral O}_2 \text{ delivery} = \text{CBF (C}_a \text{O}_2 - \text{C}_v \text{O}_2) \quad (1.7)
\]

Where \( C_aO_2 \) and \( C_vO_2 \) are the arterial and venous content of oxygen respectively. During the asphyxia associated with diving, there is a progressive decrease in \( C_aO_2 \). Therefore, there are two possible mechanisms that can maintain the delivery of oxygen to the brain during diving. First, an increase in CBF can compensate for a decreased \( C_aO_2 \) by increasing flow with a decreased \( O_2 \) content. Second, utilization of venous blood by decreasing \( C_vO_2 \) and widening the arterial-venous content difference can maintain cerebral \( O_2 \) delivery during asphyxia. Irving *et al.* (1935) were among the first to suggest that oxygen might be conserved for vital organs, such as the brain, during diving. Evidence for this hypothesis, however, awaited the development of blood flow probes and radioactively labeled tracers to examine CBF.

The results from studies that have utilized blood flow probes vary depending on species. Bevan and Butler (1992) found that blood flow doubled through the carotid
arteries during voluntary diving in ducks, suggesting a corresponding increase in CBF. Kerem and Elsner (1973) measured cerebral venous outflow in restrained seals and found that outflow did not increase, suggesting that CBF does not increase during diving. Dormer et al. (1977) found a linear increase in flow through the internal carotid artery over the entire dive duration in voluntary diving sea lions.

Relative blood flow to the brain has been demonstrated to increase markedly during diving in a number of studies. In ducks relative flow has been shown to increase by as much as three fold during forced head immersion (Johansen, 1964; Stephenson and Jones, 1992). Jones et al. (1982) found the brain's share of CO increased fifteenfold during forced diving in muskrats, whereas, Lin and Baker (1975) found only a fourfold increase in relative flow during forced diving in the rat. In seals, the brain's share of CO during diving has been shown to range from 8 times (Zapol et al., 1979) to a remarkable 23 times control values (Blix et al., 1983). However, the major disadvantage of measuring relative blood flow is that it may not necessarily represent an absolute increase in flow if it is accompanied by an associated decrease in cardiac output. Nonetheless, all studies have shown that blood flow to the brain is at least maintained during diving.

In contrast to relative blood flow, CBF has also been determined in absolute terms during diving in a number of species. In the duck, Jones et al. (1979) determined that CBF increases steadily over time and after over 2 minutes of submergence was 8.5 times pre-dive values. They attributed the increase in CBF to the cumulative effects of both an increase in CO\textsubscript{2} and a decrease in O\textsubscript{2}. Stephenson et al. (1994) found an overall doubling of CBF during forced head immersion in ducks. In
seals, CBF increases two to three times over the control values after 10 minutes dive duration (Blix et al., 1983). Zapol et al. (1979) reported only a moderate increase in CBF during diving in restrained seals. In smaller, less accomplished mammalian divers, the results have been inconsistent. In beavers CBF was found to increase almost threefold during diving (McKean, 1982), whereas in rats, blood flow to the brain was maintained at pre-dive levels (Lin and Baker, 1975).

Few studies have examined the pattern of blood flow within the brain itself. Zapol et al. (1979) and Blix et al. (1983) examined CBF in major brain divisions, such as the pons and cerebellum, during forced diving in seals. Zapol et al. (1979) reported that brain blood flow remained relatively constant during diving, whereas Blix et al. (1983) reported a differential brain blood flow pattern, that changed with dive duration (Figure 1.7). However, variations in CBF on a smaller scale could occur during diving that may not be exposed by examining CBF changes in larger brain divisions. Recently, Stephenson et al. (1994) examined CBF in discrete brain regions during forced head immersion in ducks. They found rCBF increased approximately two fold during diving in all seventeen brain regions examined.

1.3.3. *Arterial blood gases during diving.*
Figure 1.7. Blood flow, as a percentage of control values, in large brain divisions during forced diving in the harbor seal. Cerebral blood flow did not increase in any brain division until 10 minutes of diving [From Blix et al. (1983)].
Upon submersion, the basic problem facing air-breathing animals is the continuous depletion of oxygen stores and the progressive rise in carbon dioxide concentration. As dive time is extended, the changes in the concentration of carbon dioxide and oxygen are even more pronounced. In ducks, $\text{PaCO}_2$ increased from a predive level of 30 mmHg to 44 mmHg, 20 to 72 seconds into the dive (Jones et al., 1979). After two minutes of diving, $\text{PaCO}_2$ reached 53 mmHg. Opposite changes occur in $\text{PaO}_2$ during diving. After 2 minutes of diving in ducks, $\text{PaO}_2$ decreased from a predive value of 90 mmHg to 32 mmHg (Jones et al., 1979). In mammals, the time course for the change in blood gases during diving is similar to that in ducks. In beavers, after 2 minutes of forced submersion $\text{PaCO}_2$ increased from a predive level of 44 mmHg to nearly 59 mmHg. In addition, arterial oxygen tension decreased from a predive value of 83 mmHg to 30 mmHg after 2 minutes. The changes in arterial blood gases during diving are summarized in Figure 1.8.

The magnitude of the increase in $\text{PaCO}_2$ during diving likely results in a corresponding increase in CBF (Figure 1.3). Similarly, $\text{PaO}_2$ decreases after 2 minutes of diving to a level that is below the threshold to induce an increase in CBF (Figure 1.4). However, the reactivity of cerebral blood vessels to oxygen is possibly attenuated, since cerebral blood vessels may already be near maximal dilation in response to hypercapnia.

1.3.4. *The rat as a diving mammal.*
Figure 1.8. Arterial blood gases during diving in ducks (open symbols) and beavers (closed symbols). The partial pressure of oxygen (\(P_{\text{aO}_2}\)) decreased throughout the dive, suggesting a progressive hypoxemia [A]. Whereas, the partial pressure of carbon dioxide (\(P_{\text{aCO}_2}\)) increased upon submersion, indicating a progressive hypercapnia [B]. Source of data [Jones et al., (1979); McKean, (1982)].
Laboratory rats exhibit a marked cardiovascular response to diving, similar to that of small aquatic mammals such as muskrat and mink (Lin and Baker, 1975; McCulloch et al., 1997). This most likely reflects the fact that laboratory rats descend from semiaquatic wild *Rattus norvegicus* (Jackson, 1982). Wild rats living along the banks of the Po River have been observed naturally diving and feeding on molluscs found on the river bottom (Parisi and Gandolfi, 1974). Further, laboratory rats can be easily trained to dive for food (Galef, 1980).

During both forced and voluntary diving in conscious rats, arterial blood pressure is maintained constant in the face of a rapidly initiated and intense bradycardia (Lin and Baker, 1975; McCulloch et al., 1997). Voluntary diving in rats has been shown to produce an immediate and sustained 83% reduction in heart rate (McCulloch et al., 1997). In response to the decreased heart rate, TPR has been shown to increase over fourfold during diving (Lin and Baker, 1975). This suggests that TPR increases proportionally to the bradycardia-driven fall in cardiac output, as in more specialized mammalian divers.

The distribution of cardiac output during forced stationary dives in rats has been investigated. During diving, blood flow decreased by 99% in the tail and 50% in the gastrocnemius muscle. In fact, blood flow was reduced to all systemic tissue, except for the coronary, cerebral and bronchial circulations (Lin and Baker, 1975). This study demonstrates that rats, like more specialized divers, exhibit circulatory adjustments that conserve oxygen stores for the brain during diving.
1.4. RATIONALE AND OBJECTIVES OF THE STUDY.

1.4.1. Rationale.

The cardiovascular response to diving has been investigated in great detail (see references in Section 1.3.1. "Cardiovascular adjustments during diving"). The vast majority of studies have been on animals that have been restrained and forcibly submerged. The distribution of cardiac output has been shown to be different in freely diving ducks (Bevan and Butler, 1992), but this has not been demonstrated in mammals. Furthermore, the compromise between the exercise response and the diving response has been addressed (Butler, 1982; Millard et al., 1973), but not investigated in any detail.

Whole brain blood flow during diving has been determined in a number of studies (see Section 1.3.2. "Cerebrovascular adjustments during diving."). The major drawback from these studies is that CBF has only been investigated globally or in large brain divisions. There have been no studies to date that have investigated the pattern of blood flow in small brain regions, such as individual nuclei, during a mammalian diving response. A different pattern of blood flow in smaller brain regions will not be exposed by examining blood flow globally. Furthermore, very few studies have investigated the cerebrovascular response to diving in a freely moving, thinking animal.
The laboratory rat is an appropriate model to examine the cardiovascular and cerebrovascular components of the diving response (see Section 1.3.4. "The rat as a diving mammal"). In addition, the neuroanatomy of the rat is well known due to the availability of rat brain anatomical atlases (Paxinos and Watson, 1986). A knowledge of the neuroanatomy of the rat is essential to identify small brain regions, such as nuclei, in rCBF studies.

The regulation of CBF occurs on many levels that were reviewed in Section 1.2. "Regulation of Cerebral Blood Flow." Therefore, there are potentially many inputs that could control the cerebrovascular response to diving in mammals. No study has investigated the inputs that are important in the manifestation of the cerebrovascular response to diving in mammals.

1.4.2. Objectives.

The objective of this thesis was to investigate the distribution of cardiac output and regional cerebral blood flow during a mammalian diving response. The specific objectives of each of the three research chapters were as follows:

1. To determine the relative distribution of cardiac output during voluntarily initiated diving in the rat. What is the effect of the locomotory activity associated with swimming underwater on the distribution of blood flow during a diving response? Does the brain receive an increase in its share of cardiac output?
2. To investigate the distribution of regional cerebral blood flow during voluntary diving in rats. Is there a differential perfusion pattern within the brain itself during diving? Is there an intracerebral circulatory adjustment during diving that is analogous to the oxygen conserving response that occurs peripherally? Are some brain regions susceptible to hypoxia during diving while others are hyperperfused?

3. To investigate the control of the cerebrovascular response to diving in the rat. What are the mechanisms that control rCBF during a mammalian diving response? Are humoral and neurogenic inputs involved in the cerebrovascular response to diving? If yes, what is the impact of each of these stimuli upon the distribution of rCBF?
2.0. RELATIVE DISTRIBUTION OF CARDIAC OUTPUT IN VOLUNTARILY DIVING RATS.

2.1 INTRODUCTION.

The circulatory adjustments during diving have been shown to effectively decrease blood flow to peripheral tissues in favor of increasing blood flow to the heart and brain (Johansen, 1964; Zapol et al., 1979). However, in most studies to date, the distribution of cardiac output has only been determined in restrained animals in which there is no locomotory activity. Freely diving animals have exercising muscle that may possibly demand additional blood flow. In fact it has been proposed (Butler, 1982; Millard et al., 1973) that in birds, the cardiovascular adjustments made during voluntary diving may be a composite of the exercise response, where blood flow is redistributed in favor of skeletal muscle (Butler et al., 1988), and the forced dive response, where blood flow to hypoxia-insensitive tissues is effectively shut down (Jones et al., 1979).

In support of this view, the distribution of blood flow has been examined in freely diving ducks by both direct (Bevan and Butler, 1992) and indirect measurements (Stephenson and Jones, 1992). Blood flow to active leg muscles
increased nearly fivefold compared to control levels in voluntary diving ducks (Bevan and Butler, 1992). Stephenson and Jones (1992) found that the distribution of blood flow in freely diving ducks was highly variable and depended upon the dive type. During escape dives in ducks, in which the hindlimbs were active, relative blood flow to the hindlimbs increased significantly compared to the values in resting controls. However, during forced diving, in which there was no locomotory activity, relative blood flow to the hindlimbs decreased moderately compared to the values in resting ducks (Stephenson and Jones, 1992).

A similar study has not been undertaken in diving mammals. During forced stationary dives in rats, blood flow decreased by 99% in the tail and 50% in the gastrocnemius muscle (Lin and Baker, 1975). In fact, blood flow was reduced to all systemic tissue, except for the coronary, cerebral and bronchial circulations (Lin and Baker, 1975). However, it is not known if a different distribution of cardiac output occurs in rats during unrestrained conscious diving, in which there is locomotory activity.

The objective of the study was to determine the effects of locomotory activity on the distribution of cardiac output during a dive response. The relative distribution of cardiac output in conscious rats was investigated with an imaging technique using a tracer labeled with a radioisotope, technetium-99m ethyl cysteinate dimer (\(^{99m}\text{Tc-ECD}\)). Rats were trained to voluntarily initiate their own dive and swim underwater in order to produce locomotory activity simultaneous with a dive response. To determine the effect of locomotor activity alone the distribution of cardiac output during surface
swimming was evaluated without submersion. Finally, the above results were compared to the distribution of cardiac output in rats at rest.

2.2 MATERIALS AND METHODS.

2.2.1. Surgical Procedures

Experiments were performed on thirteen male Sprague-Dawley rats (383 ± 25 g). All experimental interventions were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with guidelines of the Canadian Council on Animal Care.

Rats were anesthetized with Innovar-Vet (MTC Pharmaceuticals, Cambridge, Ont; 0.15-0.2 ml·kg⁻¹ i.m., diluted to a 10% solution in saline), after initial inhalation induction with methoxyflurane (Metofane, MTC Pharmaceuticals). Buprenorphin-hydrochloride analgesic, 0.06 mg I.M (Temgesic, Reckitt and Coleman Pharmaceuticals, Hull, England) was given after surgery. The right and left femoral veins were cannulated with silicone tubing (Baxter Scientific, McGaw Park, IL; I.D. = 0.635 mm, O.D. = 1.194 mm) connected to micro-renethane tubing (Braintree Scientific, Braintree, MA; I.D. = 0.355 mm, O.D. = 0.836 mm). Cannulae were advanced six centimeters into the inferior vena cava. The cannulae were filled with heparinized sterile saline (100 IU·ml⁻¹, Hepalean, Organon Teknika Inc., Toronto, Ont.)
and fed subcutaneously to the nape of the neck, where they were connected to hypodermic tubing (23 gauge, I.D. = 0.3302 mm, O.D. = 0.635 mm; Small Parts, Miami Lakes, FL). The tubing was previously attached using dental acrylic to a patch of propylene screen cloth (Small Parts, 500 microns) which was implanted subcutaneously. One centimeter of metal tubing was exteriorized through the skin and connected to a short length of propylene tubing (PE 50; I.D. = 0.58 mm, O.D. = 0.965 mm; Clay Adams, Parsippany, NJ) which was kinked off with a piece of larger tubing (PE 205, Clay Adams). Heperanized saline (0.3 ml, 100 IU·ml⁻¹, Hepalean). The incisions were closed with wound clips (Autoclip, Clay Adams) and left the rats to recover for 4-5 days. Experiments were only performed on animals that had full recovery of their hindlimbs after surgery.

2.2.2 Training procedure.

Rats were divided into three groups before the surgery was performed for the measurement of blood flow distribution. In the control group (N=5) rats were left undisturbed in a cage. In the surface swimming group (N=4) rats swam in a large plastic cylinder for 1 minute. In the underwater swimming group (N=4) rats were trained to dive underwater through a maze constructed of Plexiglas (Figure 2.1). Water temperature was room temperature (22-24°C) in both swimming groups.

The dive training procedure has been described in detail elsewhere (McCulloch et al., 1997). Briefly, rats were trained daily for approximately two weeks. The initial
Figure 2.1. Schematic drawing of the dive tank that was used in recording pulsatile arterial blood pressure from voluntarily diving rats. The tank (70x45x25 cm) was constructed of Plexiglas. Vertical pieces inserted into the tank created a maze consisting of three channels. The rats were trained to swim underwater, in a path as depicted by the arrow. [From McCulloch et al., (1997)].
training procedure began by placing a rat in the maze and allowing the rat to swim freely on the water surface to an exit area. This familiarized the rats with the maze and enabled them to determine the location of the exit area, a ramp leading to a raised platform. The dive training started with the rats diving under a piece of Plexiglas in order to swim the rest of the maze. Once the rat dove under this barrier, it could continue on to the raised platform, and was allowed to spend time grooming. The underwater swimming distance was gradually increased by adding horizontal sub-surface Plexiglas pieces to the maze during training sessions. These were slotted to accommodate the trailing venous cannulae during an experiment. The full length of the underwater pathway was 1.3 m and trained rats accomplished the swim in 5 s (approximate underwater swim speed = 0.26 m/sec). To extend underwater duration, the exit to the platform was blocked while the rat was submerged, causing the rat to retrace its path in the maze. The sub-surface swimming was extended to a maximum of 50 seconds, which was within the maximum duration recorded for diving rats (75 seconds; unpublished observation). Once the rat was enclosed within the diving maze, the rat swam back and forth along the maze pathway up to 10 times. The procedure to extend underwater dive times was repeatedly performed on the rats during the training period. A similar procedure has been used to extend dive duration in tufted ducks (Stephenson et al., 1986). Experimental dives were similarly voluntarily initiated, but terminated by lethal injection of sodium pentobarbital (Somnotol, MTC Pharmaceuticals) after 50 s.
2.2.3. Validation of Technique.

$^{99m}$Tc-ECD is a stable, lipophilic complex which can cross all cell membranes and the intact blood-brain barrier by passive diffusion (Robinson, 1995). $^{99m}$Tc-ECD is metabolized by an esterase from a lipophilic compound to a polar metabolite, which in the case for the brain, is trapped by the blood brain barrier (Walovitch et al., 1989; Knudsen et al., 1994). $^{99m}$Tc-ECD is used widely as a brain blood flow tracer since washout of metabolites is slower from the brain than from other organs (Leveille et al., 1989). The suitability of $^{99m}$Tc-ECD to assess the relative distribution of cardiac output needed to be validated. Therefore, in preliminary experiments time/activity curves were measured to assess the rate of washout and redistribution of the tracer in specific regions of interest (ROI).

The whole body distribution of $^{99m}$Tc-ECD was examined during the first five minutes post-injection in two groups of anesthetized rats. Rats were anesthetized with Innovar-Vet (MTC Pharmaceuticals), after initial inhalation induction with methoxyflurane (Metofane, MTC Pharmaceuticals). Rats were fitted with two femoral vein cannulas (see Section 2.2.1 “Surgical Procedures.”) for injection of the tracer and a lethal dose of sodium pentobarbitol. In the first group ($N=4$), rats were killed one minute post-injection, whereas in the second group ($N=3$), rats were killed five minutes post-injection. Each rat was injected with 2 mCi of $^{99m}$Tc-ECD in 0.2 ml saline into a femoral vein cannula. Injection lasted 3-4 seconds, after which, 0.6 ml saline was flushed through the cannula. Rats were imaged in the Department of Nuclear Medicine, Royal University Hospital, Saskatoon. To determine the distribution of $^{99m}$Tc-ECD,
dynamic planar whole body images were acquired on a Maxi Camera II (GE Medical System, Milwaukee, Michigan) interfaced with a A² Medtronic computer (Ann Arbor, Michigan). Resolution was 1.5 cm and the picture matrix was composed of 128 x 128 pixels. Whole body counts were determined by drawing a ROI surrounding the entire body. The image was then divided into ROI's representing the head, front limbs, hindlimbs, thorax and abdomen. Image acquisition occurred at the rate of 1 frame/sec for the first minute post-injection and then changed to 1 frame/30sec, which was reflected as a break in the time axis (Figure 2.2).

Figures 2.2A and 2.2B show time-activity curves of $^{99m}$Tc-ECD in specific ROI's in the two groups of rats, respectively. In both figures, $^{99m}$Tc-ECD concentration peaked in each ROI within the first 30 seconds post-injection and remained at that level throughout the measurement period. There was no significant redistribution of the tracer during the measurement period. If $^{99m}$Tc-ECD distributes with blood flow to tissue within the first 30 seconds, and remains in the perfused tissue, then the distribution pattern of the acquired image must represents the peripheral perfusion pattern during the first minute post-injection. These results are in agreement with other studies, in which $^{99m}$Tc-ECD concentration peaks within 30 seconds in the arterial blood (Knudsen et al., 1994; Greenberg et al., 1994) and was taken up by all the tissues that were investigated (Vallabhajosula et al., 1989). Importantlly, although the tracer was injected on the venous side of the circulation, $^{99m}$Tc-ECD is not absorbed appreciably by lung tissue (Holman et al., 1989; Leveille et al, 1989; Vallabhajosula et al., 1989).
Fig 2.2. Time-activity curves of a bolus injection of $^{99m}$Tc-ECD into two groups of anesthetized rats. In both figures, $^{99m}$Tc-ECD concentration peaked in each ROI within the first 30 seconds post-injection and remained at that level throughout the measurement period. (A) death after 1 minute; (B) death after 5 minutes.
2.2.4. *Experimental Imaging Protocol.*

Relative whole body blood flow was evaluated by injecting $^{99m}$Tc-ECD into the systemic circulation via a venous cannula in rats that were resting, surface swimming or swimming underwater. In the underwater swimming group the complex was injected immediately after submersion. In all groups, the distribution and metabolism of $^{99m}$Tc-ECD were stopped after 50 seconds by lethal injection of 1.0 ml sodium pentobarbital (Somnotol, MTC Pharmaceuticals) into the contralateral venous cannula. The distribution of $^{99m}$Tc-ECD was stopped after 50 seconds to allow for the tracer to distribute and peak in the arterial circulation. After lethal injection the rats were refrigerated until image acquisition occurred (see Section 2.2.3. "Validation of Technique."). Static planar whole body images were acquired in order to determine the total counts in each ROI. The geometric mean for posterior and anterior counts in each ROI were determined and expressed as a percentage of total body counts and termed relative blood flow (%). The distribution of $^{99m}$Tc-ECD was color-coded from a gray level scale and displayed as a color image.

2.2.5. *Radiopharmaceuticals.*

A kit was used (Neurolite, DuPont Pharma Inc., Billerica, Mass.) for preparation of $^{99m}$Tc-ECD. Because of the short half-life (6.02 hr) of the isotope,
$^{99m}$Tc- ECD was prepared on the morning of an experiment. ECD was labeled with 100 mCi of $^{99m}$Tc and the labeling efficiency was tested by thin layer chromatography using Baker Flex Silica Gel IB-F (Phillipsburg, New Jersey) eluted with ethyl acetate. The labeling efficiency of the $^{99m}$Tc-ECD kit was consistent with manufacturer's recommendations (93%). The average amount of $^{99m}$Tc activity injected was 2.25 ± 0.2 mCi.

2.2.6. **Statistical Analysis.**

Values reported in Figures 2.2 and 2.4 are means ± standard error (SE). Statistical analyses were performed with a computer package (SYSTAT, Systat, Evanston, IL). Since percentages form a binomial distribution, rather than a normal distribution, an arcsine transformation was performed on the data in figure 4 (Zar, 1984). The resultant data was subsequently analyzed with one-way ANOVAs with repeated measures with significance reached when $P < 0.05$. In the case of significant F-values, Tukey's honestly significant difference (HSD) *a posteriori* tests were performed to determine differences among group means.

2.3. **RESULTS.**
Figure 2.3. Pseudo-color coded images of the distribution of $^{99m}$Tc- ECD in the tissues of rats during control, swimming and diving. The vertical bar indicates relative activity (equivalent to relative blood flow) where blue indicates low and white indicates high activity. The distribution of blood flow was markedly different between the three groups of rats.
Figure 2.3 demonstrates that the typical blood flow pattern differed considerably between the three groups of rats. In resting controls (Figure 2.3A), blood flow was widely distributed throughout the whole body with the thoraco-abdominal region receiving the largest fraction of cardiac output. During surface swimming (Figure 2.3B) a different flow pattern emerges, which shifts blood towards the exercising limbs. The pattern of blood flow during underwater swimming (Figure 2.3C) changes dramatically and systemic blood flow was largely restricted to the head and thorax.

Figure 2.4 represents relative blood flow (\(\%\) ± SE) in the four ROI's. During surface swimming, relative blood flow to the front limbs of surface swimming rats (27.6 ± 0.6 %) was significantly greater than that in both resting control rats (21.0 ± 0.9 %) and those swimming underwater (21.1 ± 1.2 %). Relative blood flow to the hind limbs (33.1 ± 1.8 %) was also significantly higher in surface swimmers compared to either resting controls (19.7 ± 1.5 %) or underwater swimmers (25.6 ± 2.6 %). Relative blood flow to the front and hind limbs did not increase significantly from resting control levels during underwater swimming. There was a significantly lower relative blood flow of the thorax-abdomen region during both surface swimming (42.9 ± 2.2 %) and underwater swimming (48.4 ± 2.0 %), compared to resting controls (56.6 ± 0.5 %). During underwater swimming, relative flow to the head was significantly higher (24.7 ± 0.7 %) than in both resting controls (18.1 ± 1.5 %) and surface swimming (19.3 ± 0.9 %).
Fig. 2.4. Mean relative blood flow ± SE in four regions of interest during rest, surface swimming and diving in rats. * = Response significantly different from resting state; ** = response significantly different from other two protocols; $P < 0.05$. 
2.4. DISCUSSION.

This study utilizes a radiolabeled tracer to estimate the distribution of cardiac output in freely diving, underwater swimming rats. In contrast to studies on restrained animals, the active front and hind limbs received an increased flow during diving. In agreement with other studies, there appears to be a preferential maintenance of blood flow to the head.

2.4.1. Relative distribution of cardiac output.

Relative blood flow increased to the front and hindlimbs in surface swimming rats compared with resting controls. Absolute blood flow increases to hindlimb muscle in surface swimming ducks (Butler et al., 1988) and probably reflects an increased recruitment of muscle fibers due to increased locomotory activity (Laughlin et al., 1984). Limb muscle has been shown to be essentially ischemic during forced, stationary dives in rats (Lin and Baker, 1975), ducks (Butler et al., 1971; Heieis and Jones, 1988; Johansen, 1964; Jones et al., 1979), seals (Blix et al., 1983; Zapol et al., 1979) and muskrats (Jones et al., 1982). In this diving model, rats swam underwater at a swim-speed of approximately 0.26 m/sec. At this swim-speed, the active front and hind limbs were not rendered ischemic, which is the case in forced, stationary dives. These results are in agreement with studies on diving ducks, in which perfusion was maintained to
active hindlimb muscle during voluntary diving (Bevan and Butler, 1992; Jones et al., 1988). This suggests that the metabolic demands of exercising skeletal muscle can partially override tissue-selective vasoconstriction associated with diving. This potential conflict has been previously addressed (Bevan and Butler, 1992; Castellini et al., 1985; Hochachka, 1986; Millard et al., 1973).

Stephenson and Jones (1992) experimented on unrestrained redhead ducks and found that blood flow increased to the hindlimbs during escape dives and decreased during forced dives, in which the limb muscle is inactive. It is important to understand that during the underwater swimming protocol, rats voluntarily initiated their dive but were trapped underwater later in the dive. Previous studies have shown that the oxygen-conserving response is highly variable during voluntarily initiated trapped dives in ducks (Stephenson et al., 1986; Stephenson and Jones, 1992). The bradycardia is the same regardless of the type of dive (Lin and Baker, 1975; McCulloch et al., 1997). Further, in the present study, there was a consistent perfusion pattern that emerged in all the underwater swimming rats.

Relative blood flow to the head increased significantly during underwater swimming compared to both control and surface swimming. This increase probably reflects a fall in cerebrovascular resistance and corresponding absolute increase in cerebrovascular flow (Jones et al., 1979; Jones et al., 1982; McKean, 1982). In the current experiments perfusion to the whole head, and not the brain specifically was evaluated, due to limitations in resolution of the gamma camera images and the size of the rat brain. Relative blood flow to the brain has been found to increase during forced diving in ducks (Johansen, 1964; Stephenson and Jones, 1992), muskrats (Jones et al.,
1982), rats (Lin and Baker, 1975) and seals (Blix et al., 1983; Zapol et al., 1979).
However, it must be emphasized that an increase in relative blood flow does not
necessarily represent an absolute increase if it is accompanied by a decrease in cardiac
output.

The thorax and abdomen region received a decreased relative flow during both
surface and underwater swimming compared to resting control levels. There is a similar
shift in the distribution of cardiac output away from the gut during forced diving in
ducks (Heieis et al., 1988; Jones et al., 1979; Johansen, 1964), muskrats (Jones et al.,
1982), beavers (McKeain, 1982) and rats (Lin and Baker, 1975). Unfortunately,
because of the resolution of the images, and the relatively small size of the rats, it was
not possible to quantify the thorax and abdomen separately in this study. In images of
both surface and underwater swimming the majority of the isotope activity appears in
the thoracic rather than the abdominal area (Figures 2.3B, 2.3C). This suggests that
there may be a vasoconstriction of abdominal vascular beds in these groups.

2.4.2. Evaluation of the radiological technique.

The radiological technique used in this study has several advantages and
disadvantages for whole body imaging. $^{99m}$Tc-ECD provides a visual representation of
the whole body pattern of cardiac output in freely moving rats. From these images it
was possible to estimate relative blood flow to various anatomic regions of interest.
The primary disadvantages of the technique are its low resolution and its inability to
quantify blood flow in absolute terms. Because the gamma camera images are two-
dimensional representations of a three dimensional object, only a relative evaluation of
flow could be performed. $^{99m}$Tc-ECD is a tracer that is used clinically specifically for
brain imaging. However, this tracer was utilized for estimating regional peripheral
blood flow changes. The time-activity curves used to validate use of this tracer for
estimating peripheral blood flow demonstrated that $^{99m}$Tc-ECD is taken up into specific
ROI's and remains there without washout, at least in this measurement period.
Backflux of the tracer from tissue was further minimized by rapidly stopping blood
flow one-minute post injection. Differential tracer uptake between tissues may
represent another potential source of error in the relative blood flow values. Therefore,
these values were used primarily to make comparisons between groups. Repeated
experiments could not be performed on the same animal, although they are possible
with the macroaggregated albumin technique that also employs the $^{99m}$Tc label
(Stephenson and Jones, 1992).

2.5. SUMMARY.

The blood flow distribution patterns support the view that an oxygen
conserving response involving redistribution of blood flow occurs during voluntarily
initiated diving in rats. The active front and hind limbs were not rendered totally
ischemic during diving, demonstrating that the demands of exercising skeletal muscle
partially over-ride the peripheral vasoconstriction during asphyxic diving in conscious
rats. Furthermore, relative blood flow to the head increased during underwater swimming which supports the view that there is a preferential maintenance of blood flow to the brain.
3.0. DISTRIBUTION OF REGIONAL CEREBRAL BLOOD FLOW IN VOLUNTARILY DIVING RATS.

3.1. INTRODUCTION.

The ability of mammals to dive underwater and extend underwater dive duration is dependent upon the utilization of internal oxygen stores. Maximal oxygen utilization is achieved by a cardiovascular adaptation known as the dive response (see Section 1.3. "Cardiovascular and cerebrovascular adjustments during diving."). The dive response redistributes a decreased cardiac output away from tissues with a capacity for anaerobic metabolism, such as skeletal muscle, toward tissues sensitive to hypoxia, such as the heart and brain (Irving, 1934; Johansen, 1964; Zapol et al., 1979). The results from Chapter 2, "Relative distribution of blood flow in rats during surface and submerged swimming.", suggest that blood flow is maintained to the brain during diving. Other studies have also demonstrated that the brain as a whole is continuously perfused during diving (see Section 1.3.2. "Cerebrovascular adjustments during diving."). Few studies, however, have examined the pattern of blood flow within the cerebral circulation during diving. Zapol et al. (1979) and Blix et al. (1983) examined cerebral blood flow (CBF) in major brain divisions, such as the pons and cerebellum,
during forced diving in seals. Zapol et al. (1979) reported that brain blood flow remained relatively constant during diving, whereas Blix et al. (1983) reported a differential brain blood flow pattern, that changed with dive duration. This latter study provided evidence to suggest that there is a differential perfusion pattern within the brain itself during diving in specialized divers. A differential pattern of CBF during diving could potentially meter out CBF in response to regional variations in oxygen demand within the brain. Moreover, it is possible that some brain regions are more susceptible to hypoxic damage and therefore are preferentially perfused during periods of asphyxia. These studies only measured CBF globally or in major brain divisions, such as the pons and the cerebral cortex, and did not quantify CBF in smaller brain regions such as the spinal trigeminal nucleus and the hippocampus. Variations in CBF on a smaller scale could occur during diving that would not be exposed by examining CBF changes in larger brain divisions.

In pursuit of this idea, Stephenson et al. (1994) used a radioisotope-labeled brain blood flow tracer and quantitative autoradiography to examine rCBF during forced head immersion in Pekin ducks. They found rCBF increased approximately two fold during diving in all seventeen brain regions examined. Therefore, they concluded there is not a selective intracerebral redistribution of blood flow during diving in the Pekin duck.

The objectives of this study were to reinvestigate the hypothesis of Stephenson et al. (1994) that there is a redistribution of blood flow between brain regions during diving. This hypothesis was tested by evaluating rCBF in a conscious, voluntarily diving small mammal; the rat. Conscious rats exhibit a marked redistribution of cardiac
output in response to diving similar to that of small semi-aquatic mammals such as muskrat and mink (see Chapter 2, "Relative distribution of blood flow in rats during surface and submerged swimming."; Lin and Baker, 1975). Regional cerebral blood flow during surface-swimming without submersion was evaluated to determine if locomotor activity associated with diving had any effect on rCBF. The above results were compared to the distribution of rCBF in rats at rest.

3.2. MATERIALS AND METHODS.

3.2.1. Surgical procedures.

Experiments were performed on twenty male Sprague-Dawley rats (397.6 ± 20.2 g). All experimental interventions were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with guidelines of the Canadian Council on Animal Care.

Rats were anesthetized with fentanyl-droperidol (Innovar-Vet, MTC Pharmaceuticals, Cambridge, Ont; 0.15–0.2 ml·kg⁻¹ i.m., diluted to a 10% solution in saline), after initial inhalation induction with methoxyflurane (Metofane, MTC Pharmaceuticals). Buprenorphin-hydrochloride analgesic, 0.06 mg I.M (Temgesic, Reckitt and Coleman Pharmaceuticals, Hull, England) was given after surgery. All surgical instruments were sterilized in an alcohol-iodine solution. The right and left
femoral arteries were cannulated with micro-renethane tubing (Braintree Scientific, Braintree, MA; I.D.= 0.355 mm, O.D.= 0.836 mm). Cannulae were advanced 2.0 cm toward the abdominal aorta. The right and left femoral veins were cannulated with silicone tubing (Baxter Scientific, McGaw Park, IL; I.D.= 0.635 mm, O.D.= 1.194 mm) connected to micro-renethane tubing (Braintree Scientific, Braintree, MA). Cannulae were advanced six centimeters into the inferior vena cava. All the cannulae were filled with heparinized sterile saline (100 IU·ml⁻¹, Hepalean, Organon Teknika Inc., Toronto, Ont.) and fed subcutaneously to the nape of the neck, where they were connected to hypodermic tubing (23 gauge, I.D.= 0.3302 mm, O.D.= 0.635 mm; Small Parts, Miami Lakes, FL). The tubing was previously attached to a patch of propylene screen cloth (Small Parts, 500 microns), using dental acrylic. One centimeter of metal tubing was exteriorized through the skin and connected to a short length of propylene tubing (PE 50; I.D.= 0.58 mm, O.D.= 0.965 mm; Clay Adams, Parsippany, NJ) which was kinked off with a piece of larger tubing (PE 205, Clay Adams). The incisions were closed with wound clips (Autoclip, Clay Adams) and the rats were left to recover for 4–5 days. Experiments were only performed on animals that had full recovery of hindlimb function, indicating that collateral blood flow was sufficient to maintain hindlimb perfusion.

3.2.2. *Experimental protocol.*
Before surgery, rats were divided into three groups for the measurement of rCBF. In the resting control group \((N=7)\) rats were left undisturbed in a cage. In the surface-swimming group \((N=7)\) rats were treading water in a large plastic cylinder. In the diving group \((N=6)\) rats were trained to swim underwater through a maze constructed of Plexiglas. Water temperature was room temperature \((22–24^\circ C)\) in both swimming groups.

A detailed description of the diving training procedure has been described previously (see Section 2.2.2. "Training procedure."; McCulloch et al., 1997). Briefly, the dive training started with rats diving under a single piece of Plexiglas in order to surface swim the rest of the maze. The diving distance was gradually increased by adding horizontal sub-surface pieces to the maze during training sessions. To extend underwater duration, the exit to the surface was blocked, trapping the rat underwater. A similar procedure has been used to extend dive duration in tufted ducks (Stephenson et al., 1986). Experimental dives were similarly voluntarily initiated, but terminated by lethal injection of sodium pentobarbital (Somnotol, MTC Pharmaceuticals) after 50 s.

One arterial cannula was attached to a pressure transducer (type 4-327-C, Beckman Instruments, Sciller Park, IL.) and the pulsatile signal was connected to a cardiotachometer (Beckman type 9857B) to monitor heart rate. Pulsatile arterial pressures and heart rate were recorded on a chart recorder writing on rectilinear coordinates (Beckman R511A). The second arterial cannula was connected to a preweighed heparinized 5.0 ml syringe attached to an infusion/withdrawal pump (Harvard Syringe Infusion Pump 22, Ealing Scientific, St. Laurent, PQ; 0.4 ml/min). One venous cannula was connected to a 1.0 ml syringe containing the radioactive blood
flow tracer, the other to a syringe containing 1.0 ml of pentobarbitol (Somnotol, MTC Pharmaceuticals; 65 mg/ml).

3.2.3. Measurement of regional cerebral blood flow.

The brain blood flow tracer \(^{14}\)C-N-isopropyl-\(p\)-iodoamphetamine (IMP) (NEN, Boston, Mass.) was selected to quantify rCBF. The specific activity was 44.7 mCi/mmol. IMP is extracted 100% during first pass in the brain capillaries with a brain washout t\(_{1/2}\) of 318 sec (Winchell et al., 1980). Therefore, the experiment was performed using a modification of the indicator-fractionation technique, first described by Goldman and Sapirstein (1973). IMP is ideally suited for studies in which rCBF is expected to be in the high range, since the extraction rate of IMP remains linear even at high rates of CBF (Lear et al., 1982; Bryan et al., 1988). In all protocols a reference blood sample was withdrawn at a steady rate of 0.4 ml/min which provided a reference flow rate (\(R\) in Equation 3.2) which is necessary to determine rCBF.

Rats were given at least one hour to stabilize after all the cannula connections were made. In all groups the arterial withdrawal was started first, followed by injection of IMP into the femoral vein cannula. In the resting control and surface-swimming groups, the IMP was allowed to circulate for 40 seconds, after which, pentobarbitol was rapidly infused to cause cardiac arrest and stop the circulation of the tracer. In the diving group, the IMP circulation time was extended to 50 seconds, to ensure the peak of the tracer concentration in the arterial blood had passed during the decreased cardiac
output associated with diving bradycardia. These tracer distribution times were chosen based on arterial radioisotope-dilution curves that have previously been determined in conscious rats (Lin and Baker, 1975). Lin and Baker (1975) determined that the peak of the radioisotope-dilution curve is reached within 15 seconds in control rats (heart rate = 411 ± 11 beats-min), whereas the peak of the dilution curve is not reached until approximately 30 seconds during forced diving in rats (heart rate = 118 ± beats-min). Therefore, the radioisotope circulation time was adjusted in the diving group from 30 to 50 seconds to reflect the cardiovascular changes which occur as a result of diving bradycardia. Based upon the above arterial circulation times for radioisotopes, the rCBF values in the current experiments reflect a limited time frame after injection of the tracer and not a 'smeared' measurement of the entire experimental period. For the resting control and surface-swimming groups, the values in the current experiment are indicative of rCBF in the time-frame of 5–15 seconds after injection, whereas for the dive group, the rCBF values are indicative of rCBF in the time-frame of 15–35 seconds into the dive.

A detailed account of the determination of rCBF has been described in detail elsewhere (Bryan et al., 1988; Stephenson et al., 1994). Briefly, rCBF is calculated according to the following relationship:

\[
\text{CBF} = C_b \sqrt{\frac{T}{t}} C_s \text{d}t ,
\]

(3.1)
where $C_b$ is concentration of the tracer in a particular brain region and $C_a$ is concentration of the tracer in arterial blood at any time $t$. The evaluation of the numerator in Equation 3.1 is determined by quantitative autoradiography and the denominator can be determined by measuring the tracer contained in the withdrawn blood during the experimental period ($T$) (Sapirstein, 1958; Goldman and Sapirstein, 1973; Van Uitert and Levy, 1978). It is important that the arterial blood withdrawal is terminated at the same time the animal is killed. However, timing errors are minimized by injecting IMP as a bolus, since the arterial concentration is very low towards the end of the experimental period (Patlak et al., 1984). The integrated arterial blood sample can be expressed in terms of rate of blood withdrawal, $R$ (ml-min$^{-1}$), and the total tracer activity in the withdrawal blood sample, $Q_A$ (disintegrations per minute, DPM):

$$\int_0^T C_a \, dt = Q_A / R,$$

$$\text{(3.2)}$$

$Q_A$ was determined by analyzing four 20 µL aliquots of blood from the reference blood sample which were weighed, solubilized (NCS II Tissue Solubilizer, Amersham, Oakville, Ont.), decolorized with 30% hydrogen peroxide and counted in a liquid scintillation counter (Beckman LS 9800). $Q_A$ was obtained as follows:

$$Q_A = C_s \times M_t / M_A$$

$$\text{(3.3)}$$
where $C_s$ is the quantity of tracer in an aliquot of blood, $M_s$ is the mass of the aliquot, and $M_r$ is the mass of the entire reference blood sample. A mean value of the four estimates was used for subsequent calculations.

At the end of the experimental period the brain was removed from the skull and rapidly frozen in isopentane (2-methyl-butane) at -50 °C. The brains were cut at 20-µm thickness and placed in contact with autoradiographic film (Kodak TMS-1 RA, Eastman Kodak Co., Rochester, NY; 18 × 24 cm) in a light-tight cassette. After a short exposure period (5-10 days) the film was developed and a gray level brain image with a spatial resolution of approximately 100 µm was produced (Greenberg, 1989). Densitometry was performed on autoradiographic images by a computer-based image analysis system (Image 1, Universal Imaging Corp., West Chester, PA, USA) The autoradiograph gray level density was converted to tissue tracer concentration using calibrated $C^{14}$-standards (American Radiolabeled Chemicals Inc., St. Louis, Missouri), which were packed with the brain slices (Appendix A). Regional cerebral blood flow was calculated in absolute terms (ml-min⁻¹·100g⁻¹), and rates of blood flow were pseudo-color coded and displayed as a color image (Figure 3.1). To positively identify brain nuclei, the 20 µM brain slices were stained with neutral red, which stains for Nissl bodies in neurons. The stained slides were then compared to a stereotaxic brain atlas (Paxinos and Watson, 1986) to identify specific brain regions.

3.2.4. Estimation of cardiac output.
Cardiac output (CO) was determined using the 'reference sample' technique. Blood was withdrawn from the femoral artery at a rate of 0.4 ml/min during the experimental protocol. Cardiac output was determined from the equation:

\[
CO = \frac{0.4C_i}{\int_0^t C_a \, dt},
\]

(3.4)

where \(C_i\) is the total counts of tracer injected, determined by the principle described in Equation 3.3. Therefore, cardiac output was determined by dividing the withdrawal rate by the fraction of injected tracer in the 'reference sample.' A potential error in the determination of cardiac output is the possibility that IMP is not extracted during first pass in peripheral tissues, which would result in an overestimation of cardiac output.

3.2.5. *Statistical Analysis.*

All values reported in the text and figures are grand means ± standard error (SE); Heart rate (HR; beats-minutes\(^{-1}\)) and mean arterial blood pressure (MABP; mmHg) were measured for each animal in all protocols. If only one of the two arterial cannulas was patent on the day of the experiment, a blood pressure tracing was taken before the experiment for determination of HR and MABP. HR and MABP were determined by calculating HR and MABP at 5 second intervals and then averaging the
values for the entire experimental period. MABP was calculated from pulsatile blood pressures traces (diastolic plus one-third pulse pressure). Stroke volume (SV; ml) and total peripheral resistance (TPR; mmHg·ml⁻¹·min⁻¹) were calculated by substituting MABP, cardiac output (CO) and HR into the equations:

\[
MABP = CO \times TPR \quad (3.5)
\]

and

\[
CO = HR \times SV. \quad (3.6)
\]

All cardiovascular variables represent approximately the same time frame as the rCBF values in the experimental period as described above. Regional cerebral blood flow (ml·min⁻¹·100g⁻¹ wet brain tissue) was determined in 33 brain regions from two separate measurements. Grand means were calculated by averaging the rCBF values in specific brain regions from all animals in a protocol. Hindbrain brain regions known to be involved in the integration of the dive response (Blix and Folkow, 1984) were specifically chosen for analysis. A sampling of higher brain regions was chosen based on functional neuroanatomy. For presentation purposes brain regions were grouped into divisions based upon function (basal ganglia and thalamus, limbic system, and primary cortical regions) except for the hindbrain, which was grouped according to anatomical location since the hindbrain consists of a multitude of smaller functional regions. Global cerebral blood flow (CBF; ml·min⁻¹) was estimated by using the equation:
CBF = mean rCBF x brain mass,

(3.7)

where mean rCBF represents the non-weighted average of all 33 brain regions measured in each animal. The average of the 33 brain regions was assumed to reflect flow throughout the whole brain, since the regions spanned from posterior to anterior. Since the brain had to be rapidly frozen upon removal, brain mass was calculated by using a correlation (Equation B.1; Appendix B) between rat body mass and rat brain mass from previously published data (Zeman and Innes, 1963). Cerebral vascular resistance (CVR; mmHg·ml⁻¹·min⁻¹) was determined by substituting CBF and MABP into the equation:

\[ MABP = CBF \times CVR. \]  

(3.8)

Statistical analyses were performed with a computer package (SYSTAT, Systat, Evanston, IL.). The data were analyzed with one-way analysis of variance (ANOVA) with significance reached when \( P < 0.05 \) (Zar, 1984). In the case of significant F-values, Tukey's honestly significant difference \textit{a posteriori} test were performed to determine differences among group means.

3.3. RESULTS.
Figure 3.1. Autoradiographic images of coronal sections through the rostral portion of the hippocampus, thalamus and cerebral cortex in resting control (A) and a voluntarily diving rat (B). Images have been pseudo-color coded to calibrated regional cerebral blood flow (rCBF) values. During diving, rCBF increased to all brain regions except the globus pallidus, hypothalamus and amygdala.
CONSCIOUS RESTING CONTROL

REGIONAL CEREBRAL BLOOD FLOW (ml/min/100g)

VOLUNTARILY INITIATED DIVE
Figure 3.2. Original chart recording of pulsatile blood pressure and heart rate in a conscious voluntarily diving rat swimming through an underwater maze. Downward deflection of event marker signifies bolus injection of cerebral blood flow tracer and start of arterial blood withdrawal. Injection of tracer and withdrawal of arterial blood did not interfere with cardiovascular variables throughout the dive. Upward deflection of event marker signifies lethal injection of pentobarbitol.
Table 3.1. *Cardiovascular variables in resting control, surface-swimming and diving rats.*

<table>
<thead>
<tr>
<th></th>
<th>Resting control (N = 7)</th>
<th>Surface swimming (N = 7)</th>
<th>Conscious diving (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats·minutes(^{-1}))</td>
<td>408.4±16.7</td>
<td>455.2±11.9</td>
<td>116.6±6.7**</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>120.1±6.7</td>
<td>131.0±4.9</td>
<td>157.4±4.3**</td>
</tr>
<tr>
<td>CO (ml·min(^{-1}))</td>
<td>181.4±13.2</td>
<td>268.6±33.5*</td>
<td>55.8±8.0**</td>
</tr>
<tr>
<td>TPR (mmHg·ml(^{-1})·min(^{-1}))</td>
<td>0.68±0.1</td>
<td>0.57±0.1</td>
<td>2.98±0.4**</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>0.45±0.0</td>
<td>0.55±0.1</td>
<td>0.51±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; N, no. of animals. Cardiovascular variables reflect approximately the same time period as rCBF measurements (see Section 3.2.3. "Measurement of regional cerebral blood flow and cardiac output."). HR, heart rate; MABP, mean arterial blood pressure; CO, cardiac output; TPR, total peripheral resistance; SV, stroke volume. ** Response significantly different from resting control and surface-swimming; * response significantly different from resting control; \( P<0.05 \).
3.3.1. *Cardiovascular response to surface swimming and diving.*

Heart rate decreased immediately upon voluntary submersion and remained at that level throughout the dive period (Figure 3.2). Injection of the tracer during submersion did not alter blood pressure or heart rate. Cardiovascular variables measured in the three groups of rats are presented in Table 3.1. During diving, there was a significant fall in HR (408.4 ± 16.7 to 116.6 ± 6.7 beats-minutes\(^{-1}\)) which decreased cardiac output by 69.2 % (181.4 ± 13.2 to 55.8 ± 8.0 ml-min\(^{-1}\)) from resting control values. All estimated cardiac output values were moderately higher than values previously determined by microspheres or thermodilution techniques (Coleman et al., 1984). TPR (0.68 ± 0.1 to 2.98 ± 0.4 mmHg·ml\(^{-1}·min\(^{-1}\)) increased by over 4-fold during diving, leading to a significant increase in MABP (120.1 ± 6.7 to 157.4 ± 4.3 beats-minutes\(^{-1}\)) from both resting control and surface-swimming. There was no significant difference in SV between the three groups of rats (Table 3.1). During surface-swimming, HR and SV increased slightly, but not significantly, resulting in a significant increase in cardiac output from resting control (268.6 ± 33.5 ml-min\(^{-1}\) and 181.4 ± 13.2 ml-min\(^{-1}\), respectively).

3.3.2. *Global cerebrovascular response to surface swimming and diving.*

Figure 3.3 presents the global cerebrovascular variables during control, surface-swimming and diving. During diving CBF increased significantly (7.9 ± 0.8 ml-min\(^{-1}\))
from both resting control (4.7 ± 0.4 ml·min⁻¹) and surface-swimming (5.4 ± 0.3 ml·min⁻¹). There was a significant 1.7 fold increase in CBF during diving compared to control values. CVR significantly decreased during diving (20.5 ± 1.3 mmHg·ml⁻¹·min⁻¹) from both resting control (25.9 ± 1.5 mmHg/ml/min) and surface-swimming (26.4 ± 1.3 mmHg·ml⁻¹·min⁻¹). Therefore, the increase in CBF during diving is due primarily to a corresponding 20.9% decrease in CVR compared to control values. The brain’s share of cardiac output increased over fivefold during diving (13.6 ± 1.5%) compared with both resting control (2.6 ± 0.1%) and surface-swimming (2.1 ± 0.2%).

3.3.3. *Regional cerebrovascular response to surface swimming and diving.*

Regional cerebral blood flow was determined in thirty-three brain regions (Figs. 3.4–3.7). During diving, rCBF increased significantly to 29 of the 33 brain regions examined, compared with both resting control and surface-swimming values (Figures 3.4–3.7). During diving, rCBF increased significantly from both resting control and surface-swimming values in all regions of the hindbrain and thalamus that were examined (Figures 3.4 and 3.5). There was a slight, but statistically insignificant increase in flow to the caudate putamen-posterior (CPu-P), globus pallidus (GP), hypothalamus (H) and amygdala (A) during diving compared with both resting control or surface-swimming values (Figures 3.5 and 3.6). All primary cortical regions significantly increased rCBF during diving compared to resting control values, except the inferior colliculus (IC), which was only significantly different from the surface-
Figure 3.3. Cerebral blood flow (CBF; A), % of cardiac output to the brain (% of CO; B) and cerebrovascular resistance (CVR; C) in resting control, surface-swimming and diving rats. Values are means ± SE. During diving CBF (A), the % of CO to the brain (B) and CVR were all significantly different from both resting control and surface-swimming values. ** Response significantly different from resting control and surface-swimming; \( P<0.05 \).
Figure 3.4. Regional cerebral blood flow (rCBF) values in hind brain regions of resting control, surface-swimming and diving rats. Values are means ± SE. During diving rCBF increased significantly from both resting control and diving in all regions. Sp5C, spinal trigeminal nucleus-caudal part; Sp5I, spinal trigeminal nucleus-interpolaris part; Sp5O, spinal trigeminal nucleus-oral part; Gr, gracile nucleus; Cu, cuneate nucleus; Sol, nucleus of the solitary tract; 12, hypoglossal nucleus; Lrt, lateral reticular nucleus; IO, inferior olive; 7, facial nucleus; Cb, cerebellum. ** Response significantly different from resting control and surface-swimming; P<0.05.
Figure 3.5. Regional cerebral blood flow (rCBF) values in the basal ganglia and thalamic nuclei in resting control, surface-swimming and diving rats. Values are means ± SE. During diving rCBF did not increase significantly to the posterior portion of the caudate putamen (CPu-P) and the globus pallidus (GP), but increased significantly to all regions of the thalamus compared with both resting control and surface-swimming values. CPu-A, caudate putamen-anterior; VPM/VPL, ventral posteromedial and ventral posterolateral thamic nuclei; Hb, habenular nucleus; LD, laterodorsal thalamic nucleus; MD, mediodorsal thalamic nucleus; AVVL, anteroventral thalamic nucleus. ** Response significantly different from resting control and surface-swimming; P<0.05.
Figure 3.6. Regional cerebral blood flow (rCBF) values in the reticular formation (MdV, MdD) and limbic brain regions (Ent, Hi, H, A, Cg) of resting control, surface-swimming and diving rats. Values are means ± SE. During diving rCBF increased significantly from both resting control and surface-swimming in all regions except the amygdala (A) and the hypothalamus (H). MdV, medullary reticular nucleus, ventral part; MdD, medullary reticular nucleus, dorsal part; Ent, entorhinal cortex; Hi, hippocampus; Cg, cingulate cortex. ** Response significantly different from resting control and surface-swimming; $P<0.05$. 
Figure 3.7. Regional cerebral blood flow (rCBF) values in primary cortical brain regions of resting control, surface-swimming and diving rats. Values are means ± SE. During diving rCBF increased significantly from both resting control and surface-swimming in all regions of the motor-somatosensory cortex (Fr, FL, HL and Par), the occipital cortex (Oc) and the auditory cortex (DC and IC). Fr, frontal cortex; FL, forelimb area of the cortex; HL, hindlimb area of the cortex; Par, parietal cortex; DC, dorsal cochlear nucleus; IC, inferior colliculus. ** Response significantly different from resting control and surface-swimming, * response significantly different from resting control, † response significantly different from surface-swimming; P<0.05.
swimming values (Figure 3.7). In surface-swimming rats, rCBF to the fore limb motor area (FL) of the cerebral cortex was the only significantly different brain region compared to resting control values (Figure 3.7). The largest absolute difference in rCBF (diving minus resting control) occurred in the entorhinal cortex (230 ml·min⁻¹·100g⁻¹) whereas the smallest difference was in the hypothalamus (9 ml·min⁻¹·100g⁻¹).

3.4. DISCUSSION.

This study measures, for the first time, the detailed distribution of rCBF in a conscious, voluntarily diving mammal. In 29 of 33 brain regions examined, rCBF increased significantly during diving despite a profound decrease in cardiac output associated with diving bradycardia. Only some regions of the basal ganglia (CPu-P and GP) and limbic areas (H and A) did not increase rCBF significantly during diving compared with both resting control and surface-swimming. Unequal regional participation in the global cerebrovascular response to diving suggests that there may be underlying cerebrovascular mechanisms producing differential changes in CVR. These results therefore differ somewhat from those of Stephenson et al. (1994), who found all brain regions increased flow significantly during forced diving in the Pekin duck.
3.4.1. *Global cerebrovascular response to diving.*

There was a significant 1.7-fold increase in absolute CBF during diving compared to control values. This indicates that the brain’s share of cardiac output increased over fivefold during diving. However, the estimate for CBF included brain regions composed primarily of gray matter. Perfusion to white matter has been shown to be significantly less than to gray matter (Edvinsson *et al.*, 1993). Therefore, it is possible that CBF overestimates blood flow since this value does not include blood flow to white matter in its derivation. These results agree with other studies on ducks (*Jones et al.*, 1979) and beavers (McKean, 1982) which found CBF to increase significantly during forced head immersion. The results differ from a previous study by Lin and Baker (1975), which determined the distribution of cardiac output during forced head immersion in rats. They found blood flow to the brain remained at predive levels during forced head immersion, despite a marked peripheral redistribution of cardiac output. Lin and Baker (1975), however, stated that the radioisotope which they used, $^{137}$caesium ($^{137}$Cs), is diffusion limited across the blood brain barrier and therefore potentially underestimates CBF values.

The increase in CBF during diving is primarily due to a corresponding 20.9 % decrease in CVR. Coexisting with the large decrease in CVR, was a fourfold increase in TPR that matched the decrease in cardiac output associated with diving bradycardia. These results show that the cardiovascular and cerebrovascular changes associated with mammalian diving are opposite in direction. During diving, TPR increases and CVR
decreases. The overall result is an absolute increase in CBF despite the profound decrease in cardiac output associated with diving bradycardia.

The large increase in TPR produced a significant increase in MABP during diving above both resting control and surface-swimming values. Changes in perfusion pressure do not normally result in alterations in CBF due to the ability of the cerebral circulation to autoregulate (Kuschinsky, 1988). Generally, the autoregulatory mechanism keeps CBF constant by vasodilating in response to decreased arterial pressure, and vasoconstricting in response to increased arterial pressure (Figure 1.2; Edvinsson et al., 1993). In the rat, the lower and upper limits of cerebrovascular autoregulation by MABP are approximately 50 and 150 mmHg, respectively (Hernandez et al., 1978). In this study, MABP increased to an average 157.4 ± 4.3 mmHg during diving, a perfusion pressure just outside the autoregulatory limits. However, hypercapnia has been demonstrated to shift the CBF autoregulation curve to the left (Raichle and Stone, 1972; Paulson et al., 1990). A curve shift to the left would increase CBF linearly over a lower range of blood pressures (< 150 mmHg). This suggests that the ability of the cerebral vasculature to autoregulate may be partially lost in the dive and increased perfusion pressure could make a minor contribution to the increase in CBF during diving in the rat.

3.4.2. The stimulus for the global decrease in cerebrovascular resistance during diving.
Previous studies have implicated carbon dioxide (CO\textsubscript{2}) as a potential mediator of cerebral vasomotion during diving in sea lions (Dormer et al., 1977), seals (Blix et al., 1983) and ducks (Jones et al., 1979; Stephenson et al., 1994). These investigators found flow to the brain increased linearly with dive duration, suggesting a possible link between CBF and the progressive hypercapnia associated with diving. Numerous studies have clearly demonstrated that hypercapnia elicits a marked vasodilation in the cerebral circulation (for a review, see Edvinsson et al., 1993). In almost all studies, CBF increases steeply in response to increased CO\textsubscript{2} (Figure 1.3). This suggests that progressive hypercapnia during diving possibly produces widespread cerebrovasodilation. During asphyxic diving, however, as CO\textsubscript{2} is increasing, arterial oxygen is also decreasing. Therefore, it is possible that hypoxia also stimulates a decrease in CVR. The reactivity of the cerebrovasculature in response to hypoxia is significantly less than to hypercapnia (Figure 1.4; McDowall, 1968), indicating that the majority of the decrease in CVR during diving is likely due to hypercapnia and not hypoxemia.

3.4.3. Stimuli producing differential cerebrovascular changes during diving.

The results from this study raise the possibility that not all brain regions participate equally in the global cerebrovascular response to diving, suggesting that there may be underlying mechanisms producing differential changes in CVR during diving. A number of stimuli could potentially modify the global cerebrovascular
response to conscious diving. Hypercapnia itself could be the first of these stimuli. It is generally accepted that cerebrovascular resistance is highly sensitive to changes in arterial carbon dioxide. Numerous studies have clearly demonstrated that hypercapnia elicits a marked vasodilation in the cerebral circulation (for a review, see Edvinsson et al., 1993). Regional alterations in CBF have also been reported during both hypercapnia and acute asphyxia (Shapiro et al., 1980; Goplerud et al., 1989). Therefore, it is possible that hypercapnia itself has the ability to produce a differential change in CVR during diving.

Second, stimulation of the trigeminal nerve or ganglion has been shown to produce regional variations in CBF (Goadsby and Duckworth, 1987) and decrease carotid arterial resistance (Lambert et al., 1984). In mammals, trigeminal stimulation is necessary for the manifestation of the cardiac response to diving (Drummond and Jones, 1979; McCulloch et al., 1995; McCulloch et al., 1997), whereas the role of trigeminal innervation in diving bradycardia varies considerably between bird species (Jones and Purves, 1970; Furilla and Jones, 1986). Thus it is possible that stimulation of the trigeminal nerve during mammalian diving mediates regional variations in CVR.

Third, the sympathetic component of the mammalian dive response normally produces peripheral vasoconstriction. This neural outflow could possibly act upon cerebral blood vessels. The cerebral circulation has a well-developed sympathetic innervation (for a review see Edvisson et al., 1993). Furthermore, there is evidence of a differential distribution of sympathetic nerve fibers on the cerebrovasculature (Edvinsson and Owman, 1977). The caudate nucleus and hippocampus brain regions have been shown to have an extensive vascular sympathetic innervation (Edvinsson,
1975; Edvisson and Lindvall, 1978). There is evidence to suggest that innervation distribution in the cerebral vessels parallels the degree to which sympathetic nerve stimulation produces an alteration in cerebral perfusion (Sercombe, 1975). If efferent sympathetic outflow acts differentially upon the cerebrovasculature during diving, regional variations in CBF could result.

Lastly, the variability of the capillary density in different regions of the rat brain could constrain regional increases in CBF during diving (Klein et al., 1986). In the rat brain, capillary density has been shown to vary significantly between brain regions (Gobel et al., 1990). These investigators reported that the capillary density of the inferior colliculus (IC), and some cortical areas, was higher than the capillary density of the hippocampus (H) and caudate nucleus. This anatomical constraint may be responsible for the blood flow-limitation that was observed in some regions of the basal ganglia and limbic system during diving in this study.

3.5. SUMMARY.

This study has demonstrated that the brain is preferentially perfused during conscious diving in the rat. An overall 1.7-fold increase in cerebral blood flow occurs during diving, despite a profound decrease in cardiac output associated with the dive response. A detailed examination of the regional distribution of cerebral blood flow suggests that almost all brain regions are hyperperfused during diving. Some regions of the basal ganglia and limbic system did not increase flow significantly during diving,
suggesting that not all brain regions participate equally in the global cerebrovascular response to diving. The stimuli producing the global cerebrovascular changes during conscious diving remain to be elucidated, although an obvious stimulus is progressive hypercapnia. A number of stimuli have been suggested to potentially modify the global cerebrovascular response to conscious diving. Of these stimuli, trigeminal input is a likely candidate, since this input has been demonstrated to be necessary in the cardiac response to diving in small mammals (McCulloch et al., 1997) and has been shown to elicit regional alterations in CBF (Goadsby and Duckworth, 1987). However, this input must operate within the powerful anatomical constraints of differential capillary density within the brain.
4.0. CONTRIBUTION OF HYPERCAPNIA AND TRIGEMINAL STIMULATION TO CEREBROVASCULAR DILATION DURING SIMULATED DIVING.

4.1. INTRODUCTION.

Many studies have demonstrated that the brain as a whole is continuously perfused during diving (see Section 1.3.2. “Cerebrovascular adjustments during diving.”). The results of Chapter 3, “Distribution of regional cerebral blood flow during voluntarily initiated diving in the rat.”, demonstrated that regional cerebral blood flow (rCBF) increased markedly during diving due primarily to a corresponding decrease in cerebral vascular resistance (CVR). Only some regions of the basal ganglia (caudate-putamen and globus pallidus) and limbic areas (hippocampus and amygdala) did not increase rCBF significantly during diving. Since some brain regions did not participate in the intracerebral increase in blood flow, the cerebrovasodilatory response to diving in rats may have both a humoral component mediating a global fall in CVR and a neural component mediating differential changes in CVR.

The objective of the present study was to expand upon the results of Chapter 3, “Distribution of regional cerebral blood flow during voluntarily initiated diving in the
rat."; concerning the contribution of humoral and neural inputs producing the cerebrovasodilatory response to diving in the rat. Carbon dioxide (CO₂) was investigated as a potential humoral input in cerebrovasodilatory response to diving since CO₂ has been implicated as a possible mediator of cerebral vasomotion during diving in sea lions (Dormer et al., 1977), seals (Blix and Folkow, 1984) and ducks (Johansen, 1964; Stephenson, 1994). Furthermore, numerous studies have demonstrated that CBF increases steeply in response to increased CO₂ (for a review, see Edvinsson et al., 1993). Therefore, the primary hypothesis was that the progressive increase in arterial CO₂ mediates a global fall in CVR during diving in rats.

Secondly, trigeminal stimulation was investigated as a potential neural input to the cerebrovasculature during diving, since this input has been demonstrated to be necessary in the cardiac response to diving in small mammals (McCulloch et al., 1995; McCulloch et al., 1997) and has been shown to elicit regional alterations in CBF (Goadsby and Duckworth, 1987). Moreover, the neural connections of the trigeminal system with cerebral blood vessels are so numerous, that the concept of a trigemino-cerebrovascular system has arisen (Moskowitz, 1984). Therefore, the secondary hypothesis was that trigeminal afferent input differentially modulates the global cerebrovasodilatory response to diving in rats.

In order to investigate these hypotheses, a simulated diving preparation was used in order to differentiate between the effects of carbon dioxide and trigeminal stimulation on the cerebrovasculature during diving in the rat (McCulloch and West, 1992). In the simulated diving model, the diving response was initiated by flowing water through the nasal passages (trigeminal stimulation) during expiratory apnea in
anesthetized, paralyzed, artificially ventilated rats. To investigate the first hypothesis, rCBF was measured during simulated diving in rats with pre-existing hypocapnia to remove the CO₂ stimulus (hypocapnic simulated diving). To test the second hypothesis, rCBF was measured during periods of trigeminal stimulation alone with continued ventilation (trigeminal stimulation). The results were compared from hypocapnic simulated diving, and trigeminal stimulation alone groups, to control (anesthetized, paralyzed, artificially ventilated), and normocapnic simulated diving (nasal water flow plus apnea) groups.

4.2. MATERIALS AND METHODS.

4.2.1. Surgical Preparation.

Experiments were performed on thirty male Sprague-Dawley rats (434.9 ± 16.1 g). All experimental interventions were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with guidelines of the Canadian Council on Animal Care.

A similar surgical preparation has been described previously (McCulloch and West, 1992). Briefly, rats were anesthetized with fentanyl-droperidol (Innovar-Vet, MTC Pharmaceuticals, Cambridge, Ontario; 0.15-0.2 ml·kg⁻¹ i.m., diluted to a 10% solution in saline), after initial inhalation induction with methoxyflurane (Metofane,
MTC Pharmaceuticals). Half-dose injections were given hourly to maintain anesthesia. For experiments lasting longer than 2 hours, oxymorphone (Numorphan, Dupont Merck Pharmaceuticals; 0.1 ml i.m., diluted to a 33% solution in saline) was given to ensure analgesia was maintained. Both femoral arteries were cannulated with polypropylene tubing (PE-50, Clay Adams, Parsippany, New Jersey) which was advanced 2.0 cm toward the abdominal aorta. The right and left femoral veins were cannulated with silicone tubing (Baxter Scientific, McGaw Park, Illinois; I.D.= 0.635 mm, O.D.= 1.194 mm) that was advanced six centimeters into the inferior vena cava. All the cannulae were filled with heparinized saline (Hepalean, Organon Teknika Inc., Toronto, Ontario; 10 IU·ml⁻¹). Copper leads were inserted under the skin to record the electrocardiogram (ECG) and monitor heart rate. One arterial cannula was attached to a pressure transducer (type 4-327-C, Beckman Instruments, Sciller Park, Illinois.) and recorded on a chart recorder writing on rectilinear coordinates (Beckman R511A). The second arterial cannula was connected to a preweighed heparinized 5.0 ml syringe attached to an infusion/withdrawal pump (Harvard Syringe Infusion Pump 22, Ealing Scientific, St. Laurent, Quebec; 0.4 ml/min). One venous cannula was connected to a 1.0 ml syringe containing the radioactive blood flow tracer, the other to a syringe containing the paralytic agent, d-tubocurare (Sigma, St. Louis, Missouri; 2 mg/kg i.v.). Body temperature was maintained at 37 ± 1 °C with a heating pad (Harvard Animal Temperature Control Unit, Ealing Scientific, St. Laurent, Quebec). In preparation for artificial ventilation, the rat was placed in a supine position and the trachea was exposed through a midline incision along the length of the neck. Rostral- and caudal-facing tracheal cannulas (PE-205, Clay Adams) were inserted. The caudal facing
cannula was attached to a ventilator (model CTP-930, CWE, Ardmore, PA) and the rat was paralyzed with d-tubocurare (Sigma). The oral-facing cannula was attached to a pump (model 501U/R, Watson-Marlowe, Falmouth, England) that was used to withdraw water from the cannula. Two cannulae (PE-50, Clay Adams) were inserted ~1.0 cm into the nares. Room temperature water was infused into the nares through the two cannulae using a syringe pump (Harvard Apparatus Infusion/Withdrawal Pump; 1.8 ml/min). As water flowed through the nares, water was also withdrawn from the rostral-facing cannula at a rate comparable to the infusion rate.

4.2.2. Experimental Protocol.

Cardiovascular and cerebrovascular variables were determined in four groups of rats. The control group \((N = 10)\) consisted of anesthetized, paralyzed, artificially ventilated rats \(\text{respiratory frequency (f) = 70 min}^{-1}, \text{tidal volume (Vt) = 2.6-3.8 ml}\). The normocapnic simulated dive group \((N = 7)\) consisted of anesthetized, paralyzed, artificially ventilated rats in which the dive response was elicited by flowing water into the nares (trigeminal stimulation) during concurrent apnea. The results from the above groups were compared to the results from flowing water into the nares with continuous ventilation (trigeminal stimulation alone group, \(N = 6\)) and to simulated diving after the \(P_{\text{aCO}_2}\) was reduced pre-dive by hyperventilation (hypocapnic simulated dive; \(N = 7; f = 90; Vt = 4.5-5.8 \text{ ml}\)). Arterial blood gases were determined before measurement of rCBF in all experimental groups (Table 4.1) to ensure blood gases were within the
physiological range (BGM200 blood gas meter and BC202 blood gas cell, Cameron Instrument Company, Port Aransas, TX).

4.2.3. Measurement of regional cerebral blood flow and cardiac output.

A detailed account of the determination of rCBF and cardiac output has been described in detail elsewhere (Chapter 3, Section 3.2.3. "Measurement of regional cerebral blood flow."; Bryan et al., 1988; Stephenson et al., 1994). In all groups the arterial withdrawal was started first, followed by injection of $^{14}$C-N-isopropyl-$p$-iodoamphetamine (IMP) into the femoral vein cannula. In trigeminal stimulation and simulated dive groups, the injection of IMP occurred within 2–3 seconds of a visible bradycardia on the ECG tracing (Figures 4.3 and 4.4). In the control group, the IMP was allowed to circulate for 35 seconds, after which, the rat was decapitated to stop the circulation of the tracer. In the other three experimental groups, IMP circulation time was extended to 50 seconds, to ensure the peak of the tracer concentration in the arterial blood had passed during the decreased cardiac output associated with bradycardia. These tracer distribution times were chosen based on arterial radioisotope-dilution curves that were determined previously in control and simulated diving rats (Figure 4.1). Briefly, IMP concentration in arterial blood was measured by dripping an open arterial cannula into scintillation vials during injection of IMP. The peak of the
Figure 4.1. Arterial radioisotope-dilution curves for control and normocapnic simulated diving rats. The radioisotope was injected at time 0 in both rats. During normocapnic simulated diving, the initial uptake of the radioisotope was delayed ~12 seconds due to a decreased cardiac output associated with diving bradycardia. This shifted the peak of the concentration curve from ~10-20 seconds in control to ~25-35 seconds in the simulated dive. DPM, disintegration's per minute.
arterial curve occurred approximately 10–20 seconds after injection in the control rat (heart rate = 427 beats-minutes⁻¹) whereas the peak did not appear until approximately 25–35 seconds after injection in simulated diving rats (heart rate = 104 beats-minutes⁻¹). Therefore, the circulation time of IMP was adjusted in both simulated diving groups from 35 to 50 seconds to reflect the cardiovascular changes associated with diving bradycardia. The circulation time of IMP was also adjusted to 50 seconds in the trigeminal stimulation alone group due to the occurrence of a significant bradycardia (Table 4.1). Based upon the above arterial circulation times for IMP, rCBF values in the current experiments reflect a limited time frame after injection of IMP and not a 'smeared' measurement of the entire experimental period. For the control group, the values in the current experiment are indicative of rCBF in the time-frame of approximately 10–20 seconds after injection, whereas for the dive groups, the rCBF values are indicative of rCBF in the time-frame of approximately 25–35 seconds into the dive.

4.2.4. Arterial blood gases during simulated diving.

It was not possible to determine the status of arterial blood gases during the injection of the radioisotope (IMP). Therefore separate experiments were performed (N = 5) to demonstrate that $P_{\text{a}CO_2}$ was successfully eliminated as a potential humoral input on the cerebrovasculature during hypocapnic simulated diving. Arterial blood was withdrawn and analyzed ($P_{\text{a}O_2}$, $P_{\text{a}CO_2}$ and pH₄) pre-dive and during a 10 second interval
Figure 4.2. Arterial partial pressures of O₂ and CO₂ (Pao₂ and Paco₂, respectively) and pH predive (inset) and 25-35 seconds into a dive response in normocapnic-normoxic and hypocapnic-normoxic rats. Paco₂ was successfully eliminated as a potential afferent input during simulated diving in the hypocapnic group.
(25–35 seconds) into a dive response in normocapnic and hypocapnic rats (Figure 4.2). In Figure 4.2, 30 seconds was chosen to reflect the midpoint in the arterial withdrawal interval.

4.2.5. Statistical analysis.

The statistical analysis has been described in detail in Chapter 3, Section 3.2.5. “Statistical analysis.”

4.3. RESULTS.

4.3.1. Cardiovascular responses to normocapnic simulated diving.

Normocapnic simulated diving resulted in an immediate bradycardia that was maintained throughout the entire 50 second stimulation period (Table 4.1; Figure 4.3). The physiological variables in the four groups of rats are presented in Table 4.1. Heart rate decreased an average 74.9% during the normocapnic simulated dive period compared with control values (427.6 ± 19.0 to 107.3 ± 7.3 beats-minutes\(^{-1}\)). Bradycardia resulted in a significant 55.9% decrease in cardiac output compared with control values (164.7 ± 14.1 to 64.8 ± 5.4 ml-min\(^{-1}\)). All estimated cardiac output
values were moderately higher than values previously determined by microspheres or thermodilution techniques (Coleman et al., 1984). During normocapnic simulated diving, MABP decreased below control values and was maintained at that level throughout the experimental period (Table 4.1; Figure 4.3). There was a significant increase in SV (0.36 ± 0.03 to 0.64 ± 0.02 ml) during normocapnic simulated diving compared to control values. Blood gases, determined before normocapnic simulated diving, were not significantly different from either the control or trigeminal stimulation groups (Table 4.1).

4.3.2. Cardiovascular responses to trigeminal stimulation alone.

Trigeminal stimulation alone produced an immediate and intense bradycardia that decreased in magnitude toward the end of the stimulation period (Figure 4). During the trigeminal stimulation period, heart rate significantly decreased by 39.8% (427.6 ± 20.0 to 257.3 ± 40.4 beats·min⁻¹) producing a significant 37.8% decrease in cardiac output (164.7 ± 14.1 to 102.4 ± 15.4 ml·min⁻¹) compared with control values. To counteract a decreased cardiac output, TPR increased significantly (0.6 ± 0.1 to 1.3 ± 0.2 mmHg·ml⁻¹·min⁻¹) that maintained MABP significantly higher than control values during the trigeminal stimulation period (94.3 ± 4.0 to 117.0 ± 6.4 mmHg). There was no change in SV during the trigeminal stimulation period. Blood gases, determined before trigeminal stimulation, were not significantly different from either the control or simulated diving groups (Table 4.1).
4.3.3. *Cardiovascular responses to hypocapnic simulated diving.*

Hyperventilation before simulated diving resulted in a significant increase in pH (7.57 ± 0.02) and decrease in $\text{Paco}_2$ (20.5 ± 0.7 mmHg) from control values (Table 4.1). During hypocapnic simulated diving, HR (427.6 ± 19.0 to 141.5 ± 15.6 beats-min$^{-1}$) and cardiac output (164.7 ± 14.1 to 60.5 ± 4.9 ml-min$^{-1}$) decreased significantly from control values, similarly to normocapnic simulated diving. A significant increase in TPR occurred during hypocapnic diving (0.6 ± 0.1 to 1.0 ± 0.1 mmHg·ml$^{-1}$·min$^{-1}$) compared with control values, that maintained MABP at a slightly higher level than during normocapnic simulated diving (Table 4.1). There was no change in SV during the hypocapnic dive period (Table 4.1).

4.3.4. *Arterial blood gases during simulated diving.*

Figure 4.3 shows the results of separate experiments to determine arterial blood gas changes during simulated diving in the normocapnic and hypocapnic dive groups. During simulated diving in the normocapnic group, $\text{Paco}_2$ rose from a pre-dive level of $38.6 ± 1.0$ mmHg to $48.7 ± 2.2$ mmHg, 25–35 seconds into the dive response. During a dive response in the hypocapnic group, $\text{Paco}_2$ rose from a pre-dive level of $24.8 ± 0.8$ mmHg to $35.7 ± 1.0$ mmHg, 25–35 seconds into the dive response. The changes in
PaCO₂ during simulated diving were accompanied by corresponding changes in pHₐ. PaO₂ was maintained within the normal physiological range pre-dive in both simulated diving protocols and decreased to approximately the same level during simulated diving (Figure 4.2).

4.3.5. *Global cerebrovascular response to normocapnic simulated diving.*

Figure 4.5 shows the global cerebrovascular effects of normocapnic simulated diving. During normocapnic simulated diving CBF increased a significant 1.5 fold from control values (3.4 ± 0.3 to 5.0 ± 0.5 ml·min⁻¹), whereas CVR decreased significantly compared with control values (30.1 ± 3.4 to 11.1 ± 0.8 mmHg·ml⁻¹·min⁻¹). This indicates that the increase in CBF during normocapnic simulated diving is due primarily to a corresponding 63.1% decrease in CVR. The percentage of cardiac output directed toward the brain increased over 2.8 fold during normocapnic simulated diving compared with control values (2.4 ± 0.2 to 6.9 ± 0.7 %).

4.3.6. *Global cerebrovascular response to trigeminal stimulation and hypocapnic simulated diving.*

CBF did not change from control values during either trigeminal stimulation and hypocapnic simulated diving (Figure 4.5). During hypocapnic simulated diving the
Figure 4.3. Original chart recording of a simulated diving response. Simulated diving resulted in an immediate bradycardia and hypotension that was maintained throughout the entire 50 second stimulation period. Top to bottom: time marker, ECG (electrocardiogram), pulsatile arterial blood pressure, ventilation (up, inspiration; down, expiration), event marker (down, bolus injection of IMP; up, decapitation).
Figure 4.4. Original chart recording of trigeminal stimulation alone. Trigeminal stimulation produced an immediate and intense bradycardia that subsided toward the end of the stimulation period. Top to bottom: ECG (electrocardiogram), pulsatile arterial blood pressure, ventilation (up, inspiration; down, expiration), event marker (down, bolus injection of IMP; up, decapitation).
## 4.1. Physiological variables in control, trigeminal stimulation, hypocapnic dive and simulated dive groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Trigeminal stimulation</th>
<th>Hypocapnic dive</th>
<th>Normocapnic dive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>($N = 10$)</td>
<td>($N = 6$)</td>
<td>($N = 7$)</td>
<td>($N = 7$)</td>
</tr>
<tr>
<td>HR (beats·minutes$^{-1}$)</td>
<td>427.6±19.0</td>
<td>257.3±36.9*</td>
<td>141.5±15.6†</td>
<td>107.3±7.5†</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>94.3±4.0</td>
<td>117.0±6.4*</td>
<td>60.2±4.6†</td>
<td>53.9±3.1†</td>
</tr>
<tr>
<td>CO (ml·min$^{-1}$)</td>
<td>164.7±14.1</td>
<td>102.4±15.4*</td>
<td>60.5±4.9*</td>
<td>64.8±5.4*</td>
</tr>
<tr>
<td>TPR (mmHg·ml$^{-1}$·min$^{-1}$)</td>
<td>0.61±0.06</td>
<td>1.26±0.19*</td>
<td>1.02±0.09*</td>
<td>0.79±0.05</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>0.36±0.03</td>
<td>0.43±0.06</td>
<td>0.47±0.07</td>
<td>0.64±0.02**</td>
</tr>
<tr>
<td>pH$_a$</td>
<td>7.41±0.04</td>
<td>7.36±0.07</td>
<td>7.57±0.03**</td>
<td>7.42±0.04</td>
</tr>
<tr>
<td>PaO$_2$, mmHg</td>
<td>105.3±4.3</td>
<td>118.9±5.5</td>
<td>108.1±2.6</td>
<td>96.7±7.0</td>
</tr>
<tr>
<td>Pa$_{CO_2}$, mmHg</td>
<td>34.7±0.9</td>
<td>35.8±1.7</td>
<td>20.5±0.7**</td>
<td>36.8±1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; $N$, no. of animals. Arterial blood gases were determined prior to experimental protocol. Cardiovascular variables reflect approximately the same time period as rCBF measurements (control: 10-20 s.; dive: 25-35 s.; see Section 4.2.3. "Measurement of regional cerebral blood flow and cardiac output"). HR, heart rate; MABP, mean arterial blood pressure; CO, cardiac output; TPR, total peripheral resistance; SV, stroke volume; Pa$_{O_2}$, Pa$_{CO_2}$, arterial pressure of O$_2$ and CO$_2$, respectively. ** Response significantly different from all other groups; * response significantly different from control; † response significantly from control and trigeminal stimulation; $P < 0.05$. 

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brain's share of cardiac output increased significantly compared with control values (2.4 ± 0.2 to 5.6 ± 0.4 %). This corresponded to a significant reduction in CVR during hypocapnic simulated diving compared with control values (30.1 ± 3.4 to 18.4 ± 1.3 mmHg·ml⁻¹·min⁻¹).

4.3.7. Regional cerebral blood flow during normocapnic simulated diving.

Regional cerebral blood flow was determined in thirty-two brain regions (Figures 4.6–4.9). During normocapnic simulated diving, rCBF increased significantly in sixteen of the thirty-two brain regions examined, compared with control values Table (Figures 4.6–4.9). Most regions of the hindbrain and thalamus increased rCBF during normocapnic simulated diving compared with all other groups (Figure 4.6 and 4.7). Regional CBF did not increase significantly during normocapnic simulated diving to any region of the basal ganglia or limbic system (Fig 4.7 and 4.8). Most primary cortical regions increased rCBF significantly from control values during normocapnic simulated diving (Figure 4.9). The largest absolute difference in rCBF (normocapnic simulated diving minus control) occurred in the habenular complex (Hb), in the dorsomedial aspect of the thalamus (133 ml·min⁻¹·100g⁻¹) whereas the largest decrease in rCBF occurred in the anteroventral thalamic nucleus (AVVL) (63 ml·min⁻¹·100g⁻¹).
Figure 4.5. Cerebral blood flow (CBF; A), % of cardiac output to the brain (% of CO; B) and cerebrovascular resistance (CVR; C) in control, trigeminal stimulation alone, hypoxic dive and simulated dive groups. Values are means ± SE. During normocapnic simulated diving CBF (A), the % of CO to the brain (B) and CVR (C) were all significantly different from control values. Pre-existing hypocapnia abolished the increase in CBF that was observed during normocapnic simulated diving (A). ** Response significantly different from all other groups, * response significantly different from control values; $P < 0.05$. 
Figure 4.6. Regional cerebral blood flow (rCBF) values in hind brain regions of control, trigeminal stimulation alone, hypocapnic dive and simulated dive groups. Values are means ± SE. During normocapnic simulated diving rCBF increased significantly from the all other groups in most brain regions. Pre-existing hypocapnia abolished the increase in rCBF that was observed during normocapnic simulated diving. Sp5C, spinal trigeminal nucleus-caudal part; Sp5I, spinal trigeminal nucleus-interpolaris part; Sp5O, spinal trigeminal nucleus-oral part; Gr, gracile nucleus; Cu, cuneate nucleus; Sol, nucleus of the solitary tract; 12, hypoglossal nucleus; Lrt, lateral reticular nucleus; IO, inferior olive; 7, facial nucleus; Cb, cerebellum. ** Response significantly different from all other groups; $P < 0.05$. 
Figure 4.7. Regional cerebral blood flow (rCBF) values in the basal ganglia (CPu-P, CPu-A, GP) and thalamic nuclei (VPM/VPL, Hb, LD, MD, AVVL) regions of control, trigeminal stimulation alone, hypocapnic dive and simulated dive groups. Values are means ± SE. During normocapnic simulated diving rCBF did not increase significantly to any region of the basal ganglia, but increased significantly to most regions of the thalamus. CPu-P, caudate putamen-posterior; CPu-A, caudate putamen-anterior; VPM/VPL, ventral posteromedial and ventral posterolateral thalamic nuclei; Hb, habenular nucleus; LD, laterodorsal thalamic nucleus; MD, mediadorsal thalamic nucleus; AVVL, anteroventral thalamic nucleus. ** Response significantly different from all other groups; \( P < 0.05 \).
Figure 4.8. Regional cerebral blood flow (rCBF) values in the reticular formation (MdV, MdD) and limbic brain regions (Ent, Hi, H, A, Cg) regions of control, trigeminal stimulation alone, hypocapnic dive and simulated dive groups. Values are means ± SE. Blood flow did not increase significantly to any brain region of the reticular formation or limbic system in both diving groups. MdV, medullary reticular nucleus, ventral part; MdD, medullary reticular nucleus, dorsal part; Ent, entorhinal cortex; Hi, hippocampus; H, hypothalamus; A, amygdala; Cg, cingulate cortex; $P < 0.05$. 
Figure 4.9. Regional cerebral blood flow (rCBF) values in the motor-somatosensory cortex (Fr, HL, Par), visual cortex (Oc) and auditory cortex (DC, IC) regions of control, trigeminal stimulation alone, hypocapnic dive and simulated dive groups. Values are means ± SE. During normocapnic simulated diving rCBF increased significantly from control values in all regions of the motor and somatosensory cortex. Fr, frontal cortex; HL, hindlimb area of the cortex; Par, parietal cortex; Oc, occipital cortex; DC, dorsal cochlear nucleus; IC, inferior colliculus. ** Response significantly different from all other groups, * response significantly different from control values, † response significantly different from trigeminal stimulation and hypocapnic dive groups; \( P < 0.05 \).
4.3.8. *Regional cerebral blood flow during trigeminal stimulation and hypocapnic simulated diving.*

Trigeminal stimulation alone did not produce a significant increase in rCBF from control values in any brain region, including the trigeminal nuclei themselves (Figures 4.6–4.9). During simulated diving after pre-existing hypcapnia, rCBF did not increase significantly in any of the brain regions examined (Figures 4.6–4.9).

4.4. DISCUSSION.

This is the first study to investigate the relative contribution of humoral and neural inputs on the cerebrovasculature during a dive response in a small mammal. During simulated diving CVR decreased by 63.1%, resulting in a 1.5 fold increase in CBF. Pre-existing hypcapnia abolished the cerebrovasodilatory response to simulated diving. Trigeminal stimulation alone did not produce a significant increase in rCBF from control values in any brain region. These results support the primary hypothesis that the progressive rise in arterial CO₂ produces a global fall in CVR during diving in rats. However, these results do not support a role for trigeminal input in the cerebrovascular response to simulated diving in rats.
4.4.1. *Role of carbon dioxide in the cerebrovasodilatory response to simulated diving.*

The major finding of this study is that the progressive rise in arterial CO\(_2\) decreases CVR, and increases CBF, during simulated diving in the rat. Numerous studies have clearly demonstrated that hypercapnia elicits a marked vasodilation in the cerebral circulation (for a review, see Edvinsson *et al.*, 1993). In almost all studies CBF increases steeply in response to increased CO\(_2\). This suggests that progressive hypercapnia during diving possibly produces widespread cerebrovasodilation. The reactivity of CBF in response to arterial partial pressures of oxygen (Pa\(_{\text{O}_2}\)) and carbon dioxide (Pa\(_{\text{CO}_2}\)) has been described by a sigmoid curve with the linear portion ranging between approximately 25 to 70 mmHg (Figure 1.3; Harper and Glass, 1965; Olesen *et al.*, 1971; Reivich, 1964). Therefore, as Pa\(_{\text{CO}_2}\) increases during asphyxia associated with the dive response, CBF increases linearly. Other investigators have also suggested that CO\(_2\) is the primary stimulus that increases CBF during diving in both mammals (Blix and Folkow, 1984; Dormer *et al.*, 1977) and ducks (Johansen, 1964; Stephenson *et al.*, 1994).

In this study, Pa\(_{\text{CO}_2}\) increased from a pre-dive level of 36.8 ± 1.0 mmHg to approximately 48.7 ± 2.2 mmHg during normocapnic simulated diving, a level within the linear portion of the CBF-CO\(_2\) curve. Using a previously published equation to estimate CBF reactivity in response to Pa\(_{\text{CO}_2}\), the increase in Pa\(_{\text{CO}_2}\) during normocapnic simulated diving is predicted to increase CBF by approximately 33% (Hernandez *et al.*, 1978). In this study, CBF increased by nearly 50% during simulated diving. However, the estimate for CBF included brain regions composed primarily of gray matter.
Perfusion to white matter has been shown to significantly less than to gray matter (Edvinsson et al., 1993).

During asphyxic diving, however, as CO$_2$ is increasing, arterial oxygen is also decreasing. Therefore, it is possible that hypoxia also stimulates a decrease in CVR. The reactivity of the cerebrovasculature in response to hypoxia is significantly less than to hypercapnia (McDowall and Harper, 1968; James et al., 1969). In fact, CBF has been shown to be virtually unchanged over a Pa$_{O_2}$ range of 55 to 140 mmHg, at constant Pa$_{CO_2}$ (Figure 1.4; McDowall and Harper, 1968). Below approximately 55 mmHg, however, CBF increases sharply. Therefore the effect of hypoxemia on the cerebrovasculature most likely occurs later in the dive period, only after Pa$_{O_2}$ has considerably fallen below 55 mmHg. Furthermore, although Pa$_{O_2}$ decreased to the same level in both simulated dive groups, rCBF did not increase in the hypocapnic simulated dive group to the same magnitude as in the simulated dive group. This suggests that the majority of the increase in rCBF during simulated diving is due to hypercapnia during the dive period and not hypoxemia. Jones et al. (1979) proposed that the effects of hypoxemia and hypercapnia summate to produce the marked increase in CBF observed during diving. If the additive effect of hypoxemia on CBF occurs after hypercapnia, the ability of hypoxemia to increase CBF is possibly reduced, since cerebral blood vessels may already be near maximal dilation in response to hypercapnia. Therefore, the results support hypercapnia as the primary stimulus that increases CBF during diving in the rat.

4.4.2. Blood pressure effect on cerebral blood flow during simulated diving.
Simulated diving resulted in an immediate bradycardia that was maintained throughout the entire stimulation period. However, the decrease in cardiac output was not matched by an increase in peripheral resistance, resulting in a significant hypotension during simulated diving (Figure 4.3; Table 4.1). Blood pressure is maintained above control levels in conscious, voluntary diving rats (McCulloch et al., 1997). The hypotension is likely due to the effect of the paralytic agent, d-tubocurare, on sympathetic ganglia (Hardman et al., 1996). During a mammalian diving response, an increase in sympathetic outflow produces peripheral vasoconstriction that maintains arterial blood pressure (for a review, see Blix et al., 1983). After curare administration, vasoconstriction could be partially blocked, resulting in hypotension during simulated diving. Hypotension has been shown to diminish the responsiveness of the cerebrovasculature to hypercapnia (Harper and Glass, 1965; Waaben et al., 1989). The decreased responsiveness is likely due to the autoregulatory mechanism in the cerebral circulation. Autoregulation keeps CBF constant by vasodilating in response to decreased arterial pressure, and vasoconstricting in response to increased arterial pressure (Heistad and Kontos, 1983). Therefore, cerebral vessels may have lost some of their capacity to dilate in response to hypercapnia if cerebral vessels were previously dilated in response to hypotension. This may explain why CBF did not increase in the same magnitude as during conscious diving in the rat (see Section 3.3. “Results.”).
4.4.3. *Role of trigeminal stimulation in the cerebrovasodilatory response to simulated diving.*

It was hypothesized that trigeminal input differentially modulates the global cerebrovasodilatory response to simulated diving in rats. Trigeminal stimulation alone produced profound cardiovascular changes that resulted in a decreased cardiac output (Figure 4.4; Table 4.1). However, trigeminal stimulation alone did not produce a significant increase in rCBF from control values in any brain region. Therefore, these results do not support a role for trigeminal input in the cerebrovascular response to simulated diving in rats. This result was somewhat unexpected due to a significant amount of evidence demonstrating that stimulation of the trigeminal nerve, or a ganglion associated with the nerve, produces regional variation in CBF (for a review, see Mraovitch, 1996).

There are two possible explanations why the results suggest that trigeminal stimulation does not influence CBF. First, it is possible that during trigeminal stimulation alone, the neural pathway that increases CBF is potentially inhibited at the brainstem level. The trigeminal stimulation alone protocol required continuous ventilation of the rat, indicating that afferent input from pulmonary stretch receptors (PSR) was feeding back to an integration site within the medulla during the stimulatory period (Feldman, 1986). Absence of PSR inputs, as during apnea, potentially “gates” information in the medulla during bradycardia (Lopes and Palmer, 1976). Therefore, it has been suggested that reduction of PSR afferent input is an important factor in the development of diving bradycardia (Daly, 1986). The neural pathway that increases
CBF during trigeminal ganglion stimulation occurs via a reflex that traverses the brainstem (Lambert et al., 1984). Therefore, during trigeminal stimulation alone, the neural pathway from the trigeminal ganglion that increases CBF is possibly inhibited at the brainstem level by PSR feedback. However, although trigeminal afferent input may be inhibited at the brainstem, this input would presumably increase local metabolic activity. Because neural metabolism and rCBF are normally tightly coupled, it would be expected that a metabolically driven increase in rCBF would occur to the trigeminal nuclei (McCulloch, 1988). Regional CBF did not increase significantly in any of the trigeminal nuclei during trigeminal stimulation. This raises the possibility that either metabolic activity in the trigeminal nuclei did not increase appreciably increasing rCBF, or that the IMP tracer technique is insensitive to small metabolically driven changes in rCBF.

A second explanation why trigeminal stimulation did produce CBF changes, is that the trigeminocerebrovascular pathway was not elicited in response to water flow (trigeminal stimulation). Substance P (SP) and calcitonin gene-related peptide (CGRP) are both vasoactive neuropeptides in the trigeminocerebrovascular system and produce cerebrovasodilation (Edvinsson et al., 1981; McCulloch et al., 1986). These peptides co-exist in capsaicin-sensitive unmyelinated C-fibers innervating the nasal mucosa that may be nociceptive in nature (Stjarne et al., 1989). If water flow through the nasal cavity was not sensed as a painful stimulus, then it is possible the trigeminocerebrovascular pathway was not activated during the trigeminal stimulation protocol.
4.5. SUMMARY.

This study examined the contribution of humoral (carbon dioxide) and neural (trigeminal stimulation) inputs in the cerebrovasodilatory response to simulated diving in the rat. These results provide evidence to suggest that the decrease in CVR during diving in small mammals is driven primarily by progressive hypercapnia associated with asphyxia. Although trigeminal stimulation is necessary for the cardiac component of the mammalian dive response, there is no evidence to suggest that this input has any role in the differentially modulating the cerebrovascular response to diving.
5.0. GENERAL DISCUSSION.

5.1. DISTRIBUTION OF CARDIAC OUTPUT AND CEREBRAL BLOOD FLOW DURING VOLUNTARY DIVING.

5.1.1. Distribution of cardiac output during diving.

In the first part of this thesis the distribution of cardiac output was estimated using the radiolabeled tracer $^{99m}$Tc-ECD. Using this tracer, the whole body distribution of blood flow in a freely diving small mammal was measured for first time. In contrast to studies on forced, restrained animals (Jones et al., 1982; Lin and Baker, 1975; McKean, 1982), active front and hind limbs received an increased relative flow during underwater swimming. These results are in agreement with studies on diving ducks, in which perfusion was maintained to exercising muscles during voluntary diving (Bevan and Butler, 1992; Jones et al., 1988). In this study, since the active front and hind limbs were not rendered totally ischemic, the demands of exercising skeletal muscle prevail over the peripheral vasoconstriction during diving. One possible mechanism that could over-ride the strong sympathetic signal during diving, is the accumulation of vasoactive
metabolites in exercising skeletal muscle. These results suggest that the distribution of blood flow during diving is not fixed, but can be modified in reaction to metabolizing tissue. The conflict between exercising skeletal muscle and the dive-associated peripheral vasoconstriction has been previously addressed (Bevan and Butler, 1992; Castellini et al., 1985; Hochachka, 1986; Millard et al., 1973), however, due to the difficulty of studying freely diving animals, few investigators have attempted to examine this potential conflict. The diving model used in this thesis provided a means with which to investigate whole body blood flow in a freely moving animal, a significant advantage over studying other diving animals.

Importantly, there was a preferential maintenance of blood flow to the brain during diving. Physiologically, this makes sense, in a closed system with decreasing arterial oxygen, the delivery of oxygen can only be maintained by increasing flow. This is in agreement with other studies that also found a maintenance of brain blood flow during diving (for references see 1.3.2. “Cerebrovascular adjustments during diving.”). Therefore, the results from the first part of this thesis support the view that an oxygen conserving response involving a redistribution of blood flow occurs during voluntarily initiated diving in rats. However, the results also suggest that the distribution of blood flow during diving is not fixed, but can be modified in response to stimuli such as those present in active skeletal muscle.

5.1.2. Distribution of regional cerebral blood flow diving.
The second part of this thesis examines, for the first time, the detailed distribution of regional cerebral blood flow (rCBF) in a conscious, voluntarily diving mammal. In twenty-nine of thirty-three brain regions examined, rCBF increased significantly during diving despite a profound decrease in cardiac output associated with diving bradycardia. Only some regions of the basal ganglia (caudate putamen-posterior and globus pallidus) and limbic areas (hypothalamus and amygdala) did not increase rCBF significantly during diving. These results therefore differ somewhat from those of Stephenson et al. (1994), who found all brain regions increased flow significantly during forced diving in the duck. Globally, the increase in rCBF equates to an overall 1.7 fold increase in CBF, suggesting that the brain as a whole is hyperperfused during diving. This is in agreement with other studies on ducks (Jones et al., 1979) and beavers (McKean, 1982) in which CBF increased significantly during forced head immersion. However, the results differ from a previous study on rats by Lin and Baker (1975), that found blood flow to the brain remained at predive levels during forced head immersion.

The results from this thesis demonstrate that the brain is preferentially perfused during conscious diving in the rat. Furthermore, the results show that the cardiovascular and cerebrovascular changes associated with mammalian diving are opposite in direction. During diving, total peripheral vascular resistance increases, while at the same time, cerebral vascular resistance decreases. Therefore, during diving, blood flow is shifted away from regions of high resistance and redirected toward parallel regions of low resistance. Fortunately, the region with lowest resistance during
diving is the brain, resulting in an increase in absolute blood flow to the brain during diving.

5.1.3. *Role of hypercapnia and trigeminal stimulation in the cerebrovascular response to diving.*

The unequal regional participation in the global cerebrovascular response to diving suggested that there may be underlying cerebrovascular mechanisms producing differential changes in resistance. Therefore the last part of this thesis investigated the relative contribution of humoral and neural inputs on the cerebrovasculature during a dive response. A simulated diving preparation was used in order to differentiate between the effects of particular inputs on the cerebrovasculature. There are potentially many inputs modulating the cerebrovascular response to diving; this thesis examined the two most obvious, hypercapnia and trigeminal stimulation. Carbon dioxide (CO$_2$) was investigated as a potential humoral input in cerebrovasodilatory response to diving since CO$_2$ has been implicated as a possible mediator of cerebral vasoemotion during diving in sea lions (Dormer *et al.*, 1977), seals (Blix and Folkow, 1984) and ducks (Johansen, 1964; Stephenson, 1994). Furthermore, numerous studies have demonstrated that CBF increases steeply in response to increased CO$_2$ (for a review, see Edvinsson *et al.*, 1993). Trigeminal stimulation was investigated as a potential neural input to the cerebrovasculature during diving, since this input has been demonstrated to be necessary in the cardiac response to diving in small mammals.
(McCulloch et al., 1995; McCulloch et al., 1997) and has been shown to elicit regional alterations in CBF (Goadsby and Duckworth, 1987). Moreover, the neural connections of the trigeminal system with cerebral blood vessels are so numerous, that the concept of a trigeminocerebrovascular system has arisen (Moskowitz, 1984). Pre-existing hypocapnia abolished the cerebrovasodilatory response to simulated diving. This result provided evidence to suggest that the decrease in cerebrovascular resistance during diving in small mammals is driven primarily by progressive hypercapnia associated with asphyxia. Others have also suggested that hypercapnia is the primary stimulus that increases CBF during diving, but it has never been investigated (Blix and Folkow, 1984; Dormer et al., 1977; Johansen, 1964; Stephenson et al., 1994).

The results from this thesis suggest that trigeminal input does not play any role in differentially modulating the cerebrovascular response to diving. This result was unexpected due to a significant amount of evidence demonstrating that stimulation of the trigeminal nerve, or a ganglion associated with the nerve, produces regional variation in CBF (for a review see, Mraovitch, 1996). Although trigeminal stimulation is necessary for the cardiac component of the mammalian dive response (McCulloch et al., 1995), the results from this thesis do not support a role for trigeminal input in the cerebrovascular response to simulated diving in rats.

5.1.4. Limitations of the study.
The major limitation with the imaging technique used in this thesis, is that the blood flow measurements only represent a "snapshot" in time. It is possible that the efferent manifestation of the diving response changes over time. In particular, CBF has been shown to increase steadily over the duration of a dive (Blix et al., 1981; Dormer et al., 1977; Jones et al., 1979). This suggests that the absolute value of CBF can be different, depending on when in the dive the measurement was taken. Therefore, in this thesis, it is important to understand that the cerebrovascular hemodynamics during diving were characterized only at a specific time. Unfortunately, due to the short dive duration in the rat, it was not possible to investigate the CBF changes at a further time in the dive. Arterial blood gases progressively change throughout the length of the dive (see Section 1.3.3. "Arterial blood gases during diving."). Therefore, the stimulus that drives the increase in CBF during diving is potentially greater in longer dives. It would be possible to investigate CBF at longer dive times in a more accomplished diver, such as a muskrat, that can withstand a longer underwater duration.

5.2. CONTROL OF CEREBRAL BLOOD FLOW DURING DIVING.

5.2.1. Is carbon dioxide the only stimulus to increase cerebral blood flow during diving?
Although the results from this thesis suggest that carbon dioxide primarily drives the increase in CBF during diving, it is possible that other control mechanisms are involved. However, this thesis has ruled out one of these candidates; trigeminal input. Other potential inputs that possibly contribute to the manifestation of the cerebrovascular response to diving will subsequently be discussed.

**Sympathetic innervation.** The sympathetic component of the mammalian dive response normally produces peripheral vasoconstriction. Sympathetic fibers distribute to vessels throughout the cerebral circulation and there is a regional heterogeneity in the extent of innervation to small brain divisions (see references in Section 1.2.1.1. “Sympathetic innervation.”). Since sympathetic outflow is a major component of the mammalian diving response, it is logical that this outflow possibly affects cerebral blood vessels during diving. However, the result of sympathetic stimulation of cerebral vessels is vasoconstriction; an effect that would decrease CBF and be counterproductive to increasing dive duration. The sympathetic system seems to be important in modifying the autoregulatory response of the cerebral circulation to arterial pressure (see Section 1.2.3.1. “The cardiovascular system and the regulation cerebral blood flow.”). Recent evidence also suggests that the sympathetic system may be involved in limiting the increase in CBF that occurs during both hypercapnia and hypoxia (Busja and Heistad, 1984; Kissen and Weiss, 1989). Therefore, if sympathetic innervation has any involvement in the cerebrovascular component of the diving response it most likely functions to attenuate the hypercapnia-driven decrease in cerebrovascular resistance.
Parasympathetic innervation. The profound bradycardia during a mammalian diving response is the result of parasympathetic outflow to the heart (Drummond and Jones, 1979). Since the parasympathetic system contributes nerves to the cerebral circulation (see references in Section 1.2.1.2. "Parasympathetic innervation."), it is possible that there is parasympathetic outflow to the brain during diving. Stimulation of parasympathetic nerves to the brain, increases CBF (Goasby, 1989; Goadsby, 1991). However, since the parasympathetic pathway does not play any role in modulating the CBF response to hypercapnia (Busija and Heistad, 1982; Goadsby, 1991), this input is unlikely to be important during diving. These fibers may, however, contribute to cerebral autoregulation during decreasing perfusion pressure (see Section 1.2.3.1. "The cardiovascular system and the regulation cerebral blood flow."). Arterial blood pressure is preserved during conscious diving but is partially lost during simulated diving (see Section 4.3.1. "Cardiovascular responses to normocapnic simulated diving."). This suggests that parasympathetic input is potentially important during the hypotension associated with simulated diving in the rat. However, in conscious animals, blood pressure is maintained at predive levels throughout the dive, suggesting that this input is probably insignificant in freely diving animals.

Intrinsic neural innervation. The diving response is a manifestation of autonomic efferent output from the medulla (Blix and Folkow, 1984). The NTS is the principal site of integration of cardiovascular and respiratory afferents (Andresen and Kunze, 1994). Therefore it is logical that this region is important in the integration of afferent
inputs during the diving response. In fact, diving bradycardia is attenuated by application of selective antagonists into the NTS (Huang et al., 1991). In addition, the C1 area is a major site of origin for sympathetic efferent information (Reis and Iadecola, 1989). Presumably, during diving there is an increased activity in these neurons, leading to an increased sympathetic activation. Therefore it is possible that both of these nuclei are stimulated during the integration of the diving response. Both of these regions have been demonstrated to increase CBF upon stimulation (see Section 1.2.1.4. “Intrinsic neural innervation.”) and could therefore contribute to the cerebrovasodilatory response to diving. The possible involvement of these nuclei in the cerebrovascular response to diving may be worth further investigation.

**Metabolic input.** It is well established that the products of cerebral tissue metabolism can increase local CBF (see Section 1.2.2. “Metabolic control of cerebral blood flow”). In diving animals, the brain continues to metabolize substrate, producing potential vasoactive mediators that possibly leads to local variations in CBF. In particular, active brain regions involved with the integration of the diving response may demand increased blood flow to match the increased neural activity. Therefore, it is conceivable that vasoactive metabolites contribute to cerebrovasodilation during diving. Such a contribution to the cerebrovascular response to diving is difficult to elucidate, however, if there is any effect of these vasodilators, they are likely overpowered by the hypercapnic signal of systemic origin.
**Arterial Hypoxemia.** During the ensuing asphyxia that is associated with diving, aerobically metabolizing tissues, such as the brain, eventually contribute to the development of a progressive hypoxemia. A decrease in arterial oxygen has been shown to increase CBF. However, the reactivity of the cerebrovasculature in response to hypoxia is significantly less than to hypercapnia (see Section 1.2.3.2. "The respiratory system and the regulation cerebral blood flow."). CBF increases in response to decreasing levels of oxygen only after oxygen tension has fallen quite low (~55 mmHg). Therefore the effect of hypoxemia on the cerebrovasculature most likely occurs later in the dive period, only after \( P_{\text{a}O_2} \) has considerably fallen to a level below approximately 55 mmHg. Jones et al. (1979) proposed that the effects of hypoxemia and hypercapnia summate to produce the marked increase in CBF observed during diving. If the additive effect of hypoxemia on CBF occurs after hypercapnia, the ability of hypoxemia to increase CBF is possibly reduced, since cerebral blood vessels may already be near maximal dilation in response to hypercapnia. Therefore, it is unlikely that hypoxemia plays a major role in eliciting the cerebrovasodilation during diving.

**Arterial blood pressure.** In most animals, arterial blood pressure remains relatively unchanged during diving (Blix and Folkow, 1984, Butler and Jones, 1982). Changes in arterial pressure do not normally result in alterations in CBF due to the autoregulatory ability of the cerebral circulation (see Section 1.2.3.1. "The cardiovascular system and the regulation cerebral blood flow."). However, CBF autoregulation can be modified by hypercapnia (Paulson et al., 1990). During
hypercapnia, cerebral blood vessels may lose the ability for autoregulation at lower arterial pressures. Therefore the hypercapnia associated with diving could lower the upper limit of autoregulation, resulting in linear increases in CBF at lower arterial pressures. This mechanism could possibly increase CBF during diving.

**Anatomical constraints.** In the rat brain, capillary density has been shown to differ significantly between brain regions (Gobel *et al.*, 1990; Klein *et al.*, 1986). These investigators reported that the inferior colliculus and some cortical areas had a higher capillary density than the capillary density of the hippocampus and caudate nucleus. This anatomical constraint may be responsible for the blood flow-limitation that was observed in some regions of the basal ganglia and limbic system during diving in this study. Therefore, although there may be a strong input from hypercapnia to elicit cerebrovasodilation, this signal may be limited by the ability of brain regions to increase blood flow if capillary density is low.

In summary, it is evident that numerous inputs potentially modulate the distribution of CBF during diving. However, if any of these inputs are involved in the cerebrovascular response to diving, this thesis has shown that they are likely overwhelmed by the powerful vasodilatory hypercapnic signal of systemic origin. The physiological outcome of this CO₂-driven cerebrovasodilation is a prolonged maintenance of oxygen and glucose delivery, substrates critical for brain survival and therefore underwater endurance.
5.2.2. **Future experiments.**

This thesis has shown that hypercapnia is primarily the stimulus for the widespread decrease in cerebral vascular resistance during diving. There is little doubt that the cerebrovasculature has a significant response to hypercapnia, however, the precise mechanism(s) by which CO₂ dilates cerebral blood vessels remain to be elucidated. Recently, a number of studies have investigated the role of nitric oxide (NO) in the regulation of the cerebral circulation (for reviews see Faraci and Brian, 1994; Iadecola *et al*., 1994; Brian *et al*., 1996). In particular NO has been implicated in mediating the cerebrovasodilatory response to hypercapnia. Therefore, in view of recent evidence suggesting that NO is involved in hypercapnic cerebrovasodilation, further experiments could explore if NO mediates the cerebrovascular response to diving. This could be investigated by using NO synthase blockers to potentially attenuate the cerebrovasodilation during diving. These experiments could possibly demonstrate the that NO is involved in the cerebrovasodilation associated with diving.

If NO is linked to the cerebrovasodilation during diving, the question remains as to the cellular source of NO within the brain itself. Previously, NO production was thought to be limited to the endothelium, however, in the brain, NO is produced by isoforms of NO synthase in endothelium, neurons, perivascular nerves and astrocytes (Bredt *et al*., 1990). Therefore, another potential area of research would be to identify the source(s) of NO in the brain during diving. This could be accomplished by
selectively blocking the isoforms of NO synthase. This study could possibly determine the location of NO that is released in response to asphyxia during diving.

In common with other mammals, humans show a diving response, consisting of bradycardia, an increase in arterial pressure and a decrease in cardiac output (for review see Lin, 1982). The diving response in humans is qualitatively similar to that in marine mammals but typically less marked. A detailed distribution of cardiac output during breath-hold (BH) diving in humans has not been determined. However, limb blood flow has been shown to decrease 50% during BH immersion, suggesting that there is a redistribution of cardiac output during diving (Lin, 1982). Furthermore, available data suggests that peripheral resistance increases during BH diving in humans, although not to the same degree as in other mammals (Hong et al., 1971). Recently, Jiang et al. (1994) measured the velocity in the common carotid artery during BH immersion in humans. Assuming blood flow in the common carotid artery reflects blood flow to the brain, they suggested that cerebral blood flow (CBF) increases in response to BH immersion in humans. Anecdotal evidence also suggests that the brain is preferentially perfused in humans during BH diving. Gooden (1992) suggested that cerebrovasodilation in response to diving is the brain’s primary defensive mechanism by which near-drowning victims do not suffer brain damage. Despite the above evidence suggesting that humans, like other mammals, possess a cerebrovascular response to diving, there has been little or no research on the perfusion pattern of the human brain during diving. If the diving response is the defensive mechanism by which blood flow is maintained to the brain in near-drowning victims, then an examination of brain blood flow distribution during BH
diving may be beneficial in understanding the pattern of neurological injury in near-drowning victims. Moreover, it is possible that some brain regions, such as the basal ganglia (see Section 3.3.3. "Regional cerebrovascular response to surface swimming and diving."), are susceptible to hypoxic damage during periods of asphyxia diving. Therefore, another potential area of research is to determine if there is a cerebrovascular response to BH immersion in humans. Preliminary results from a recent study suggest that the brain is continuously perfused during HI (Appendix C). Furthermore, there was no alteration in the brain blood flow pattern during head immersion compared to the control pattern (Figures C2 and C3).

5.2.3. *Significance of the study.*

The cardiovascular components of the mammalian diving response have been studied for over sixty years. It has long been proposed that the physiological purpose of the diving response is to maintain blood flow and therefore oxygen delivery to those tissues that need it most, presumably the heart and the brain. Through this judicious redistribution of blood flow, diving animals can tolerate a longer dive duration than would normally be expected based upon oxygen stores. Following the development of experimental methods that enabled researchers to study freely diving animals, it became apparent that the distribution of cardiac output during diving may not have been as fixed as previously thought. The hierarchy of blood flow distribution in freely diving ducks was found to include other active tissue, such as active skeletal muscle.
Therefore, it was proposed that in freely diving animals, another component may interact with the dive response – the exercise response. In this study rats were trained to freely dive through an underwater maze; an experimental model that closely parallels freely diving animals in the wild. Although skeletal muscle has a greater ability to operate anaerobically compared to brain tissue, blood flow was not completely diverted away from the exercising skeletal tissue. Therefore, the results of this thesis demonstrate that the distribution of cardiac output during diving is not fixed to strictly favor the heart and brain, but may modified in response to stimuli such as those present in active skeletal muscle.

There is little doubt that the brain is preferentially perfused during asphyxic diving in all vertebrates. Increased CBF maintains oxygen and substrate delivery to the brain despite a reduced cardiac output and a progressively falling arterial oxygen content during diving. This study is the first attempt to investigate the distribution of rCBF in a freely diving small mammal. Previous studies limited the scope of their examination of the cerebrovascular response to diving to the global response; that is, blood flow to the brain as a whole. This study clearly demonstrates that the distribution pattern with the brain itself is not homogenous, but varies significantly from brain region to brain region. This view of the cerebrovascular hemodynamics during diving has never been demonstrated before. The results from this study show that, during diving, cerebrovascular resistance decreases and peripheral vascular resistance increases. These resistance changes during diving result in decreased blood flow to the periphery and increased blood flow to the brain. The overall result of increasing blood
flow to the brain during diving is a maintenance of oxygen delivery, and a prolongation of dive duration.

This study is the first attempt to investigate the mechanisms underlying the increase in CBF during the diving response in a small mammal. This study used a simulated diving protocol that provided a means to investigate the role of humoral and neural inputs thought to be important in diving. Under circumstances in which ventilatory drive can acquire oxygen from the environment, rCBF is closely matched to local changes in metabolic activity. Such local changes might reasonably be expected in simulated diving, because the central integration of input from cardiorespiratory receptors presumably results in increased neural activity in some brain regions, such as the NTS and trigeminal nuclei. If the coupling of rCBF to metabolic activity occurs during diving, it is evident from the results of this thesis that the large systemic increase in arterial CO₂ has the capacity to overwhelm any response from these stimuli or any other potential input.
6.0. REFERENCES.


7.0. APPENDICES.

Appendix A. Calibration of C\textsuperscript{14} standards.

In order to convert the optical density of a specific brain region on an autoradiograph to a concentration of radioactivity in absolute terms (disintegrations per minute per gram; DPM/g), a C\textsuperscript{14}-standard slide (American Radiolabeled Chemicals Inc., St. Louis, MI.) was packed along with experimental brain slices during the exposure period. The C\textsuperscript{14}-standard slide needed to be calibrated to tissue concentration by generating a standard curve relating radioactivity and optical density (Figures A1 and A2). A similar calibration procedure had been previously described (Reivich et al., 1969).

The procedure for calibration of the C\textsuperscript{14}-standard slide was as follows. Four rat brains were cut up, combined together and made into a homogenous “brain paste” totaling approximately 8.0 grams. Less than 1.0 gram of the total brain paste was used to make the first concentration point by adding a small drop of the tracer, \textsuperscript{14}C-N-isopropyl-p-iodoamphetamine (IMP) (NEN, Boston, Mass.). This was mixed well, and a small amount (<0.5 gram) was taken out in duplicate, weighed, solubilized (NCS II Tissue Solubilizer, Amersham), and counted in a liquid scintillation counter (Beckman LS 9800). The two samples were averaged to yield the known DPM/g for one point on the standard curve. Some of the brain paste was taken up into a 1.0 ml syringe and the remainder was put aside to be diluted. The syringe was centrifuged for 10 minutes, frozen overnight and the brain paste inside was cut into 20 μm circular slices. The
brain paste used for the first point on the standard curve was diluted by adding more brain paste. This was repeated eleven times to produce a range of radioactive concentrations. The 20-μm circular slices of brain paste were placed in contact with autoradiographic film (Kodak TMS-1 RA, Eastman Kodak Co., Rochester, NY; 18 × 24 cm) in a light-tight cassette. Densitometry was performed on the resultant gray level images by a computer-based image analysis system (Image 1, Universal Imaging Corp.). The range of concentrations that were in the brain pastes were found to be beyond the range of the film, and would therefore saturate the film at the higher concentrations. It is important that the length of the exposure period does not saturate the film optical density in the range of radioactivity that is to be measured, therefore, two different exposure periods were used. The slices that were in the high range of radioactivity were exposed to film for 4 days, whereas the lower radioactivity slices were exposed to the film for one week. Therefore, two standard curves were generated, a low (Figure A1) and a high concentration (Figure A2). To ensure linearity, the extrapolated values that were generated from the curves were plotted against the original manufactured concentrations on the 14C-standards (Figure A3). Once the 14C-standards were calibrated, they were packed along with the brain slices from each experiment and a standard curve, relating optical density and radioactivity, was generated for each autoradiograph. After the radioactivity in the brain region of interest was known, regional cerebral blood flow (rCBF) was calculated in absolute terms.
References.

Figure A1. Standard curve of radioactivity (DPM/g) vs. optical density (O_D) for low concentration of radioactivity in the brain slices. The first order regression relating radioactivity and O_D was (r^2 = 0.96):

\[ DPM/g = 2.94 \times 10^6 - 1.16 \times 10^4 O_D \]  \hspace{1cm} (A.1)
Figure A2. Standard curve of radioactivity (DPM/g) vs. optical density (O_D) for high concentration of radioactivity in the brain slices. The first order regression relating radioactivity and O_D was (r^2 = 0.98):

\[ \text{DPM/g} = 1.09 \times 10^7 - 4.67 \times 10^4 \text{O}_D \]  

(A.2)
Figure A3. Calibrated values in disintegrations per minute (DPM/g) vs. manufacturer’s concentration in nanocurie/gram (nCi/g) for $^{14}$C-standard slides. The first order linear regression generated a $r^2 = 0.96$. 
Appendix B. Estimation of rat brain weight.

In order to calculate cerebral blood flow (CBF), it was necessary to know the weight of the whole rat brain (Equation 3.7). However, it was not possible to weigh each brain after removal because it had to be rapidly frozen in order to avoid back diffusion of the tracer. Therefore, brain weight was estimated based on rat weight from previously published data (Zeman and Maitland Innes, 1963). A first order linear regression curve was generated relating rat body weight and brain weight (Figure B1).
Figure B1. First order regression curve of brain weight vs. body weight in rats. The regression equation relating brain weight and rat body weight was ($r^2 = 0.98$):

$$\text{brain weight} = (8.325 \times 10^{-6} \times \text{body weight}) + 1.7095 \quad (B.1)$$
Appendix C. Preliminary results from human simulated diving study.

The human simulated diving study was carried out in the Department of Nuclear Medicine, Royal University Hospital. The distribution of CBF and cardiac output were evaluated using single photon emission computed tomography (SPECT) and the brain blood flow tracer technetium-99m ethyl cysteinate dimer ($^{99m}$Tc-ECD). The distribution of CBF and cardiac output were examined in each subject during resting control and BH immersion. Each subject served as their own control. The control or baseline levels of CBF and cardiac output distribution were determined two days prior to the head immersion protocols. $^{99m}$Tc-ECD was injected as a bolus into the antecubital vein in both protocol groups. In the BH immersion groups, $^{99m}$Tc-ECD was injected immediately following head immersion. BH immersion time was verified to be a sufficient distribution time for the tracer, by determining the transit time from the antecubital vein to the internal carotid. BH immersion consisted of the subject immersing their head in a container of water for a minimum 60 seconds at 20 °C. One minute following $^{99m}$Tc-ECD injection (i.v.), whole body distribution of cardiac output was estimated by performing a whole-body scan that was acquired on a computer based image analysis system (Figure C1.). One hour after $^{99m}$Tc-ECD injection, a brain SPECT was performed (Figures C2 and C3.).
Figure C1. Whole body distribution of cardiac output during resting control (A) and breath-hold immersion (B). There was a moderate shift in blood flow away from the peripheral regions during immersion.
Figure C.2. Transverse, coronal and sagittal SPECT images of cerebral blood flow during resting control (A) and breath-hold immersion (B). There were no apparent difference in the cerebral blood flow distribution profile during immersion compared to control.
Figure C3. Transverse SPECT images of cerebral blood flow in the basal ganglia during resting control (A) and breath-hold immersion (B). There was no difference in blood flow to the basal ganglia (encircled region) during immersion compared to the control distribution.